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(57) Abstract

An *in vitro* culture of central nervous system (CNS) neuronal cells is disclosed. The culture is characterized by an absence of feeder cells or medium conditioned by feeder cells, substantially serum–free culture medium, and extended viability. Also disclosed are methods for culturing purified CNS cells under defined conditions for extended periods, as well as methods of promoting regeneration and survival of damaged CNS neurons *in vivo*, and a pharmaceutical composition for same.

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LONG-TERM SURVIVAL AND REGENERATION OF CENTRAL NERVOUS SYSTEM NEURONS

FIELD OF THE INVENTION

The present invention relates to compositions and conditions that promote survival and regeneration of central nervous system (CNS) neurons *in vivo* and *in vitro*.

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BACKGROUND OF THE INVENTION

The survival of neurons in the mammalian brain throughout the lifetime of the animal depends on continuous signalling by neurotrophic peptide factors. Both developing and adult central nervous system (CNS) and peripheral nervous system (PNS) neurons undergo apoptosis (programmed cell death) when deprived of survival signals. Survival signals are generally peptide trophic factors that are provided by neighboring cell types. The factors act by inhibiting a constitutively active program of cell death (Raff, 1992; Raff, *et al.*, 1993).

Survival control mechanisms are thought to help to match the number of pre- and postsynaptic target neurons. For instance, neurons are generally overproduced during normal development (often by at least a factor of two) and a competition between the presynaptic neurons for a limiting amount of target-derived trophic factors determines which cells live and which cells die (Cowan, *et al.*, 1984; Purves, 1988). The same survival signals that control cell survival also control survival and growth of neuronal processes and nerve terminals (Cowan, *et al.*, 1984; Campenot, 1994).

It has been shown that PNS neurons, including ventral horn motor neurons, sympathetic neurons, and dorsal root ganglion sensory neurons, may be purified and the signaling processes that promote their survival studied (*e.g.*, see review by Barde, *et al.*, 1989). The mechanisms that normally control the survival of PNS neurons have been elucidated by studying the properties of purified neurons in culture. This work has demonstrated that the survival of highly purified PNS neurons, including sensory, sympathetic, ciliary and motor neurons, can be promoted by single peptide trophic factors under serum-free conditions (e.g., see review by Barde, *et al.*, 1989). Similar work on control of CNS neurons has been largely precluded by the inability to purify such cells and by the difficulty of getting them to survive in culture. Specifically, although it has been suggested that the survival of CNS neurons depends on the presence of growth factors (*e.g.*, Hughes, *et al.*), the mere application of such factors under defined conditions was not sufficient to promote long-term survival

Several efforts currently underway are aimed at promoting regeneration after axonal injury (e.g., regeneration of retinal ganglion cells after optic nerve transection or regeneration of sensory and motor pathways after spinal cord injury), by delivering exogenous trophic factors into the brain (Schnell, *et al.*, 1994). The findings detailed herein present reasons why this approach has not succeeded and why it is unlikely to succeed in the future. Further, the present disclosure teaches methods by which such regeneration, as well as survival of pure cultures of CNS neurons under defined conditions, is promoted.

SUMMARY OF THE INVENTION

In one aspect, the invention includes a method of promoting survival of neuronal cells in the central nervous system (CNS) in a subject, particularly in regions that are degenerating by virtue of acute trauma or chronic disease. The method includes exposing the CNS neurons to one or more of a number of growth factors described herein in the presence of conditions effective to produce elevated levels of cyclic adenosine monophosphate (cAMP) in the neurons, as evidenced by ability to stimulate a cAMP-dependent protein kinase in the cells.

Growth factors may be selected from the general categories, defined herein as insulin-like growth factors, neurotrophins, and CNTF-like cytokines.

In a preferred embodiment such growth factor(s) is selected from the group consisting of insulin, brain-derived neurotrophic factor (BDNF), insulin-like growth factor 1 (IGF-1), insulin-like growth factor 2 (IGF-2), neurotrophin-4/5 (NT-4/5), ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), interleukin-6 (IL-6), basic fibroblast growth factor (bFGF), and transforming growth factor-alpha (TGF-α).

In another preferred embodiment, the method of the invention includes an activator of a cAMP dependent protein kinase, such as forskolin (or forskolin analog) or a cAMP analog, particularly a membrane permeable cAMP analog, including, but not limited to chlorophenylthio-cAMP (CPTcAMP), Sp-cAMP and 8-bromo cAMP.

In still a further preferred embodiment, neuronal cell survival is promoted by exposing the cells to a phosphodiesterase (PDE) inhibitor, such as IBMX, theophylline, caffeine or any of a number of such compounds known in the art.

In other embodiments, the foregoing "cocktail" of growth factors, cAMP elevating (and/or protein kinase activating) agents is by injection into an extracellular region surrounding the neuronal cells. In a preferred embodiment the CNS cells are retinal ganglion cells, injection of the cocktail is conveniently carried out by intraocular injection. Here, it has been shown, in experiments described herein, that a particularly effective cocktail will include brain-derived neurotrophic factor (BDNF), insulin-like growth factor 1 (IGF-1) and ciliary neurotrophic factor (CNTF). Further, it is now appreciated that it will be useful to include a PDE inhibitor in such cocktail for intravitreal injection.

In yet another embodiment, the activation of adenylate cyclase (or means to produce elevated levels of cAMP) may be accomplished by subjecting cells to depolarizing conditions, such as by electrical stimulation, ionic shifts (high potassium levels), glutamate antagonists, and the like. Thus, it would be possible, according to one aspect of this feature of the invention, to administer growth factors and PDE inhibitor by direct injection in conjunction with probe electrical stimulation of the area.

In a further related embodiment, activation of adenylate cyclase is evidenced by activation of MAP-kinase in the CNS cells, particularly as assessed by nuclear translocation of the enzyme. Methods for monitoring this phenomenon are discussed herein.

In a related aspect, the invention includes a pharmaceutical cocktail that includes the foregoing components as they relate to the present invention, in a suitable pharmaceutical excipient. It is appreciated that at least a portion of the components of the cocktail can be substituted by co-electrical stimulation, as discussed above and herein.

In yet another related aspect, the present invention includes an *in vitro* culture of central nervous system (CNS) neuronal cells. The culture is characterized by (i) an absence of feeder cells or medium conditioned by feeder cells, (ii) substantially serum-free culture medium, and (iii) short-term viability. Short-term viability is defined by (i) at least 30% cell survival after 3 days of culture, and (ii) maintenance of cell characteristics, as evidenced by the presence of a measurable cell-specific marker in the surviving cells after 3 days.

In one embodiment, the culture contains postnatal CNS neuronal cells and is further characterized by long-term viability, as defined by (i) at least 30% cell survival after 7 days of culture, and (ii) maintenance of cell characteristics, as evidenced by the presence of a measurable cell-specific marker in the surviving cells after 7 days. In a related embodiment, the culture is further characterized by very long-term viability, as defined by at least 30% cell survival after 28 days of culture.

The CNS neuronal cells in the above-described cultures may be prenatal neuronal cells or postnatal neuronal cells. For example, the cells may be glutamatergic projection neurons, such as cortical projection neurons or retinal ganglion cells. The cell-specific marker may be, for example, MAP-2 or tau.

In another aspect, the invention includes a method of growing an *in vitro* culture of central nervous system (CNS) neuronal cells. The method includes incubating the cells in a medium which (i) is substantially free of feeder cells or medium conditioned by feeder cells, (ii) is substantially serum-free, and (iii) contains a first growth factor effective to increase survival time of the cells, and growing the cells under conditions effective to stimulate cAMP-dependent protein kinases in the cells.

The first growth factor employed in the above method may be, for example, insulin, brain-derived neurotrophic factor (BDNF), insulin-like growth factor 1 (IGF-1), insulin-like growth factor 2 (IGF-2), neurotrophin-4/5 (NT-4/5), ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), interleukin-6 (IL-6), basic fibroblast growth factor (bFGF), or transforming growth factor-alpha (TGF-α).

The conditions effective to stimulate a cAMP-dependent protein kinase include pharmacological manipulations, such as exposing the cells to forskolin or membrane-permeable

analogs of cAMP, such as chlorophenylthio-cAMP (CPTcAMP), Sp-cAMP or 8-bromo cAMP. Alternatively, the conditions effective to stimulate cAMP-dependent protein kinases may include depolarizing the cells in the presence of about 200 μM to 10 mM (i.e., physiological extracellular levels) extracellular calcium. Such depolarizing may be accomplished, for example, by exposing the cells to elevated levels of extracellular potassium, exposing the cells to a glutamate receptor agonist, or by electrically stimulating the cells, for example, with an implantable stimulating electrode.

The cells suitable for culturing by the above method include glutamatergic projection neurons, such as retinal ganglion cells and cortical projection neurons.

In one embodiment, the medium employed in the above method further includes a second growth factor, which is effective to further increase the survival time of the cells. In a related embodiment, the medium contains, in addition to the first and second growth factors, a third growth factor effective to further increase the survival time of the cells. The first growth factor may be an insulin-like growth factor (e.g., insulin, IGF-1 or IGF-2), the second growth factor may be a neurotrophin (e.g., BDNF or NT-4/5), and the third growth factor may be a CNTF-like cytokine (e.g., CNTF, LIF or IL-6). Of course, the correspondence between specific classes of factors recited above, and "first", "second" and "third" growth factors, may be rearranged as desired (*i.e.*, the first growth factor may be a neurotrophin, the second growth factor may be a CNTF-like cytokine, and so-on).

In yet another aspect, the invention includes a method of extending the survival time of an *in vitro* culture of central nervous system (CNS) neuronal cells. The method includes maintaining the cells (i) in the presence of a first growth factor, and (ii) under conditions effective to stimulate cAMP-dependent protein kinases in said cells. The survival time of cells maintained as stated in this method is longer than the survival time of cells not maintained in this manner. In one embodiment, the culture is a substantially serum-free culture. In another embodiment, the culture does not contain feeder cells or medium conditioned by feeder cells.

The growth factor used with the above-described aspect of the invention may be, for example, insulin, BDNF, IGF-1, IGF-2, NT-4/5, CNTF, LIF, IL-6, bFGF, or TGF-α, and the conditions effective to stimulate a cAMP-dependent protein kinase may include any of those recited above for other aspects of the invention. The cells amenable for use with the method also include those cell types recited above.

In one embodiment, the method includes adding a second growth factor to the culture. In a related embodiment, the method includes adding a third growth factor to the culture. The first, second and third growth factors may include classes of factors as described above.

In still another aspect, the present invention includes a central nervous system neuronal cell survival factor having the following characteristics:

(i) secreted by tectal cells grown in a pure *in vitro* culture, and

(iv) capability of increasing the survival time of purified embryonic day 18 rat retinal ganglion cells plated on merosin-coated coverslips in a modified Bottenstein-Sato medium containing 5 μg/ml insulin, 5 μM forskolin, 50 ng/ml BDNF and 50 ng/ml CNTF.

The invention also includes, in another aspect, a method of promoting survival and regeneration of damaged CNS neuronal cells, such as glutamatergic projection neurons or retinal ganglion cells, in a mammalian subject. The method includes causing activation of protein kinase A in the damaged neuronal cells (*e.g.*, pharmacologically or by electrical stimulation of the cells), and delivering a first growth factor to the cells. The combination of activating protein kinase A and delivering the growth factor is effective to promote survival and regeneration of the cells. Other embodiments of the method include delivering a second growth factor, and a second and third growth factors, where the growth factors may be selected from specific classes as described above. For example, the first growth factor may be selected from the group consisting of insulin, brain-derived neurotrophic factor (BDNF), insulin-like growth factor 1 (IGF-1), insulin-like growth factor 2 (IGF-2), neurotrophin-4/5 (NT-4/5), ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), interleukin-6 (IL-6), basic fibroblast growth factor (bFGF), and transforming growth factor-alpha (TGF-α).

In one embodiment, the cells are glutamatergic projection neurons (e.g., retinal ganglion cells or cortical projection neurons), the first growth factor is selected from the group consisting of insulin, IGF-1 and IGF-2, a second growth factor is selected from the group consisting of BDNF and NT-4/5, and a third growth factor is selected from the group consisting of CNTF, LIF and IL-6.

In one aspect, the invention includes a central nervous system neuronal cell survival factor having the following characteristics: (i) secreted by mature oligodendrocytes in a pure *in vitro* culture, (ii) sensitivity to heat inactivation by boiling for 5 minutes, (iii) sensitivity to inactivation by trypsin digestion, (iv) a molecular weight greater than approximately 10 kD, and (v) application of the factor to purified cultured postnatal retinal ganglion cells increases the survival time of the cells relative to cultured cells to which the factor is not applied. Exemplary culture conditions for culturing the purified postnatal retinal ganglion cells include plating the cells on merosin-coated coverslips in a modified Bottenstein-Sato medium containing 5 μg/ml insulin, 5 μM forskolin, 50 ng/ml BDNF and 50 ng/ml CNTF.

In another aspect, the invention includes a method of promoting survival and regeneration of damaged central nervous system (CNS) neuronal cells in a mammalian subject. The method includes (i) causing activation of cAMP-dependent protein kinase in the cells, and delivering a therapeutically effective amount of the oligodendrocyte factor described to the cells. The combination of the activation of cAMP-dependent protein kinase and the delivering of the factor is

effective to promote survival and regeneration of the cells. The activation of cAMP-dependent protein kinase may include electrically stimulating the cells, such as by methods described above. The cells may be glutamatergic projection neurons, such as cortical projection neurons or retinal ganglion cells.

In specific embodiments, the method may further include delivering an effective amount of a second growth factor (e.g., an insulin-like growth factor) to the cells, delivering a therapeutically effective amount of a second and a third growth factor (e.g., a neurotrophin) to the cells, or delivering a therapeutically effective amount of a second, a third and a fourth growth factor (e.g., a CNTF-like cytokine) to the cells.

In another aspect, the invention includes a method of treating a brain disorder causing central nervous system (CNS) neuronal cell death or degeneration. The method includes delivering to the cells, a dose of the oligodendrocyte-derived neurotrophic factor described above effective to reduce neuronal cell death or degeneration. The disorder may be, for example, an acute injury to a portion of the central nervous system or a neurodegenerative disorder, such as Alzheimer's disease or amyotrophic lateral sclerosis. The disorder may also be, for example, glaucoma, or a demyelinating disorder, such as multiple sclerosis. In specific embodiments, the method may include activating a cAMP-dependent protein kinase (PKA) in the cells (e.g., by electrically stimulating the cells or by administering a pharmacological substance which, for example, acts to elevate intracellular cAMP levels or acts as a membrane-permeant analog of cAMP.

These and other objects and features of the invention will become more fully apparent when the following detailed description is read in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A, 1B, 1C, 1D and 1E show a schematic diagram of the panning procedure for purifying retinal ganglion cells.

Figure 2A shows dose-response curves of survival promoting effects of different concentrations of BDNF and CNTF on 3 day cultures of purified retinal ganglion cells (RGCs).

Figure 2B shows a bar graph indicating that combination of factors from 3 different classes produce additive effects on survival of RGCs in 3 day cultures.

Figure 3 shows that long-term survival of RGCs can be promoted by combinations of peptide trophic factors and cAMP elevating agents (survival measured at 3, 7, 14 and 28 days).

Figures 4A and 4B show the effects of cell density and basic fibroblast growth factor (bFGF) on RGC cell survival in 3 day cultures.

Figures 5A and 5B show computer-generated images of exemplary photomicrographs of purified P8 retinal ganglion cells cultured for 14 days in the absence (Fig. 5A) or presence (Fig. 5B) of a conditioning layer of mature oligodendrocytes.

Figure 6 shows the survival of purified E18 retinal ganglion cells cultured for 3 days in serum-free modified Bottenstein-Sato medium (MBS; described below) containing high insulin (5 μg/ml) and survival factors as assessed by the MTT assay.

Figure 7 shows the survival of purified E18 retinal ganglion cells after 3 days of culture above a conditioning layer of various cell types in serum-free MBS medium containing forskolin (5 μM) and plateau levels of BDNF, CNTF, and insulin.

Figures 8A and 8B show computer-generated images of the morphology of purified E18 retinal ganglion cells cultured for 3 days in serum-free MBS medium containing plateau concentrations of BDNF, CNTF, IGF-1 on merosin-coated wells in a 96-well plate with (Fig. 8B) or without (Fig. 8A) conditioned medium from E18 tectal cells.

Figure 9A shows the percent survival of purified P8 retinal ganglion cells cultured in serum-free MBS medium for 2 days in the indicated survival factors with and without forskolin or potassium chloride.

Figure 9B shows the percent survival of purified P8 RGCs cultured in serum-free MBS medium for 3 days in plateau levels of BDNF, CNTF and insulin, as well as forskolin, tetrodotoxin, kynurenic acid, NMDA and kainate (as indicated).

Figure 10 shows a computer-generated image of a field of purified postnatal retinal ganglion cells cultured in serum-free MBS medium containing BDNF, CNTF and IGF-1 for one week.

Figures 11A and 11B show a computer-generated image of a field of purified postnatal 7-day cultures of RGCs immunostained with an anti-MAP-2 monoclonal antibody (Fig. 11A) or an anti-tau monoclonal antibody (Fig. 11B).

Figures 12A and 12B show electrophysiological records of action potentials in response to depolarizing current injection (Fig. 12A) and spontaneous excitatory postsynaptic currents (Fig. 12B) recorded from purified postnatal 7-day cultures of RGCs.

Figure 13 shows the percent survival of Thy-1 positive cerebral cortical neurons purified by immunopanning from postnatal day 8 rat cerebral cortices and grown in MBS medium containing BDNF, CNTF, insulin or forskolin, individually or in combination as indicated for 3 days.

Figures 14A-14C show a computer-generated images of P8 retinal ganglion cells in culture exposed to 0, 1 and 10μM forskolin, respectively, and stained using an antibody specific for cAMP.

Figures 15A-15D show computer-generated images of purified retinal ganglion cells in culture incubated with medium alone (15A), 0.1mM IBMX (15B), glutamate +IBMX (15C) or KCl+IBMX (15D) and stained for cAMP.

Figure 16 shows a graph of the number of retinal ganglion cells exhibiting nuclear translocated MAP kinase as a function of time, in response to various factors in the absence or presence of forskolin, as indicated).

Figures 17A and 17B show computer-generated images of P8 retinas incubated with IBMX alone (17A) or IBMX+forskolin (17B) and stained for cAMP.

Figures 18A-18D show computer-generated images of P8 retinas incubated in serum-free medium for 3 hours, then exposed to no additional treatment (18A, 18B) or a cocktail of trophic factors (BDNF and CNTF), forskolin and IBMX (18C, 18D), showing immunoreactivity of nuclei to MAP kinase in the presence but not the absence of the cocktail.

Figure 19 shows a bar graph which illustrates the responsiveness, as assessed by MAP kinase translocation, of retinal ganglion cells in intact retina to trophic factors and forskolin, and blockage of activation by tetrodotoxin, curare and kynurenic acid.

Figure 20 shows a bar graph of survival of P8 retinal ganglion cells *in vivo* following axotomy and intravitreal injection of buffer (sham), BDNF alone, BDNF, CNTF and IGF-1 ("Factors"), Factors + forskolin, or factors, forskolin + IBMX, as indicated.

Figures 21(A-C) show computer-generated images of the retinal ganglion cell layer in retinas treated as described for Figure 20.

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

A "defined medium" is a culture medium used for culturing a selected sample of cells, where the identities and concentrations of all of the components present in the medium are known prior to its addition to the sample of cells. An exemplary defined medium is the serum-free modified Bottenstein-Sato medium (MBS; described below). Serum added to a cell culture medium typically renders the resulting medium undefined, since the identity and concentration of all of the serum components is not ordinarily known. Similarly, the addition of "conditioned" medium to a cell culture medium renders the resulting medium undefined, since the identity and concentration of all of the components secreted by the conditioning cells into the conditioned medium are not generally known.

The term "defined conditions", as applied to tissue culture, refers to conditions employing a defined medium to grow a sample of cells in the absence of "feeder cells" (see below) or serum. For example, purified retinal ganglion cells grown in serum-free MBS medium in the absence of glial or other feeder cells are said to be grown under defined conditions.

The term "feeder cells" as applied to a culture of neuronal cells is understood to mean non-neuronal cells present in a culture to promote the survival, differentiation, growth or viability of the cultured neuronal cells. For example, fibroblasts plated as a confluent layer for use as a substrate for neuronal cells are considered to be feeder cells. Similarly, in a mixed (unpure; see below for definition of pure) culture containing glia and neurons, the glia are considered to be feeder cells.

A culture is considered to be a "pure" culture of a single cell type if greater that approximately 99% of the cells in that culture are of that cell type. For example, a culture containing 99.5% retinal ganglion cells and 0.5% glial cells is considered to be a pure culture of retinal ganglion cells.

A culture medium is considered to be "substantially serum-free" if the concentration of serum in the medium is insufficient to result in the medium having characteristics normally associated with a serum-containing medium. For example, many "undefined" media employ 5%-15% serum to promote the growth and survival of the cells with which they are used. If the serum concentration in such a medium is dropped below a threshold level (*e.g.*, 2%), the medium may be no better at promoting cell growth and survival than a medium containing no serum. Such a medium is considered to be substantially serum-free. Of course, a medium containing no serum is also considered to be "substantially serum-free".

"Treating" a disease refers to administering a therapeutic substance effective to reduce the symptoms of the disease and/or lessen the severity of the disease.

"Promoting survival" means that cells exposed to conditions that would normally result in cell death survive for a significantly longer period of time than expected, as assessed by comparison of treated cells to control, untreated cells. Such conditions include, but are not limited to lesion of presynaptic inputs, trauma, neuronal ischemia and the like.

II. Overview of the Invention

It has been discovered in the course of experiments detailed herein that the survival of pure cultures of CNS cells is promoted by the application of trophic factors under conditions effective to stimulate cAMP-dependent protein kinase (*e.g.*, elevation of intracellular levels of cAMP). Elevation of cAMP may be accomplished pharmacologically by including, for example, forskolin (a specific activator of adenylate cyclase) or chlorphenylthio-cAMP (a cell permeable synthetic analog of cAMP) in the culture medium. Neither application of trophic factors in the absence of cAMP elevation, nor elevation of cAMP in the absence of trophic factors, was effective to promote survival of the purified CNS neurons (*e.g.*, retinal ganglion cells).

However, elevation of cAMP together with peptide trophic factors produced a dramatic enhancement of CNS neuronal cell survival. Further, direct experimental support is presented

suggesting that cAMP elevation acts to stimulate cAMP-dependent protein kinase (PKA), and that inhibition of the kinase inhibits the survival-promoting effects of cAMP elevation/trophic factor application. Accordingly, it is contemplated that the survival-promoting effects of cAMP elevation are mediated by PKA.

Surprisingly, it was found that activation of the cAMP pathway is necessary and sufficient for responsiveness to all of the survival factors tested (including BDNF, CNTF, IGF-1, NT-4/5, LIF, IL-6, bFGF, TGF-alpha and ODNF), regardless of the factor's individual molecular mechanisms of action. Additionally, it was found that this cAMP pathway could be activated by depolarizing the CNS neurons (*e.g.*, retinal ganglion cells), either by exposing them to elevated extracellular potassium or by applying glutamate agonists. With regard to promoting survival, these stimuli mimicked the effects of forskolin.

Furthermore, the ability of electrical activity (depolarization) to promote trophic factor responsiveness was entirely abolished by specific protein kinase A inhibitors, suggesting that such activity normally elevates the levels of cAMP in the CNS neuronal cells. In this regard, according to the teachings herein, CNS neurons differ from PNS neurons in that CNS neurons need to be electrically active in order to respond to their trophic factors.

In further discoveries supporting the present invention, it was found that these results were immediately applicable to central nervous system (CNS) neuronal cells *in vivo*. Thus, as described herein, CNS neurons subjected to degenerating conditions, such as axotomy or lesion of the pre-synaptic pathway, and exposed to a cocktail containing selected growth factors in combination with an activator of adenylate cyclase and/or intracellular protein kinase(s), survive significantly longer than control cells under the same degenerating conditions.

The results summarized above have a number of implications. One is that trophic factors are necessary for the survival and growth of CNS neurons and their processes. The requirement of electrical activity to respond to their factors provides a simple learning mechanism: active neurons are trophically rewarded and thus survive and grow better, whereas silent neurons and their processes atrophy and die. This is consistent with the roles of the CNS and the PNS (*i.e.*, the CNS needs to learn whereas the PNS does not).

Secondly, the present discoveries immediately suggest a reason why untreated PNS neurons can repair and regenerate axons after injury while CNS neurons cannot. While not wishing to be bound by a particular mechanism for the workings of the present invention, it is contemplated that because injured CNS neurons lose their electrical activity or become entirely silent (as their synaptic inputs have been shown to strip off quickly after injury) following injury, they lose their ability to respond to the trophic factors that are essential for their survival and regeneration. This observation may explain why CNS neurons *in vivo* do not survive or regenerate after injury.

III. The Central Nervous System

A. Anatomical Organization of the CNS

All animals belonging to the phylum Chordata have a single, dorsal nerve cord comprising the central nervous system (CNS). In mammals, this dorsal nerve chord includes the brain and the spinal cord. The CNS acts together with the peripheral nervous system (PNS), which is composed of ganglia and peripheral nerves that lie outside the brain and spinal cord, to control virtually all aspects of an animal's behavior and response to sensory stimuli. Although the central and peripheral nervous systems are separated anatomically, functionally they are interconnected and interactive (Kandel, *et al.*, 1991).

The peripheral nervous system has two divisions, somatic and autonomic. The somatic nervous system includes neurons of the dorsal root and cranial ganglia that innervate the skin, muscles and joints and provide the CNS with information about limb position and external environment. The axons of somatic motorneurons that innervate skeletal muscle and project to the periphery are often considered part of the peripheral nervous system, even though their cell bodies lie in the spinal cord (CNS).

The autonomic nervous system controls internal body functions, including the viscera, smooth muscle and exocrine glands, and consists of three parts - the sympathetic system, parasympathetic system and enteric system. The sympathetic system participates in the body's response to stress, whereas the parasympathetic system acts to restore homeostasis. The enteric nervous system controls the function of the smooth muscle of the gut.

The central nervous system receives sensory information (typically via the PNS) from sensory organs, such as the eyes, ears and receptors within the body, analyzes the information, and initiates an appropriate response (*e.g.*, moving a muscle). The CNS also initiates responses aimed at satisfying certain "drives". Some, such as the drive for survival, are basic and are initiated unconsciously or automatically ("fight or flight" response). Others are more complex, and revolve around the need to experience positive emotions (*e.g.*, pleasure, excitement) and avoid negative ones (*e.g*, pain, anxiety, frustration).

The central nervous systems consists of six main regions: (i) the spinal cord, (ii) the medulla, (iii) the pons and cerebellum, (iv) the midbrain, (v) the diencephalon, and (vi) the cerebral hemispheres. The spinal cord is the simplest and most caudal part of the CNS. It extends from the base of the skull through the first lumbar vertebra, receives sensory information from the periphery of the trunk and limbs, and contains the motor neurons responsible for voluntary as well as reflex movements. It also receives some sensory information from internal organs and is involved in the control of many visceral functions.

The medulla, pons, cerebellum and midbrain are collectively termed the brain stem. The brain stem is positioned between the spinal cord and the diencephalon, and is concerned, in part,

with sensation from the skin and joints in the face and neck, as well as with hearing, taste and balance. Motor neurons in the brain stem control the muscles of the head and neck. The brain stem also contains ascending and descending pathways that carry sensory and motor information to and from higher brain regions (Kandel, *et al.*, 1991).

The diencephalon consists of the thalamus and hypothalamus. The thalamus processes and distributes most of the sensory and motor information going to the cerebral cortex, and may regulate levels of awareness and emotional aspects of sensory experiences. The hypothalamus regulates the autonomic nervous system and hormonal secretion by the pituitary gland (Kandel, *et al.*, 1991).

The cerebral hemispheres form the largest region of the brain. They consist of the cerebral cortex (mostly cell bodies), the underlying white matter (nerve fibers), and three deep-lying nuclei - the basal ganglia, the hippocampal formation and the amygdala. The cerebral hemispheres are divided by the interhemispheric fissure and are concerned with perceptual, cognitive and higher motor functions, as well as emotion and memory (Kandel, *et al.*, 1991).

The visible part of the cerebral cortex that forms the highly convoluted surface of the cerebral hemisphere is called the neocortex, and may be divided into six distinct layers, termed I through VI. Layer I consists mostly of fibers, level II contains small pyramidal cells and level III contains large pyramidal cells. Both layers II and III project to and receive input from other cortical areas, as well as receiving input from layer IV. The pyramidal cells comprising levels II and III are almost exclusively glutamatergic, and due to the relatively distal aspect of their targets, are termed glutamatergic projection neurons.

Layer IV consists primarily of stellate cells which receive input from the thalamus and project to other layers of the cortex. Layer V contains large pyramidal cells which receive input from layer IV and project to the brainstem and spinal cord, while layer VI contains medium pyramidal cells, which receive input from the thalamus and other cortical areas and project to the thalamus. The pyramidal cells of layers V and VI are also considered glutamatergic projection neurons. In fact, nearly all projection neurons in the mammalian brain are glutamatergic projection neurons.

In addition to the brain and spinal cord, certain sensory organs, such as the retina of the eye, are considered, anatomically, to belong to the central nervous system. The retina contains five major classes of neurons (photoreceptors, bipolar cells, horizontal cells, amacrine cells and ganglion cells) arranged in an orderly layered arrangement and linked together in an intricate pattern of connections. The outer layer contains the photoreceptor cells (rods and cones), which hyperpolarize in response to light and communicate this signal through a group of interneurons (bipolar, horizontal and amacrine cells) to the ganglion cells. Ganglion cells are the output neurons of the retina, and have glutamatergic synapses (*i.e.*, they are glutamatergic projection

neurons). The axons of the ganglion cells run through the optic nerve and synapse on two subcortical targets - the lateral geniculate nucleus and the superior colliculus. It will be understood that the structure referred to as "tectum" in rats is analogous to the superior colliculus in primates; in fact, some investigators refer to the rat tectum as "superior colliculus".

B. Cells of the CNS

The two major classes of cells in the CNS are neurons and glia.

1. Neurons. Neurons, or nerve cells, carry the electrical signals that underlie he functioning of the brain. The human brain contains about 10^{11} neurons. Although the nerve cells may be classified into perhaps as many as 10,000 different types, they share many common features (nerve cells with very similar cellular properties may produce very different actions, and therefore be functionally classified as distinct types of cells, depending on their precise connections with each other and their connections with sensory receptors and muscle).

The neurons can be classified functionally into three distinct groups -- afferent neurons, motor neurons and interneurons. Afferent, or sensory neurons, carry information into the CNS for conscious perception and motor coordination, while motor neurons carry commands to muscles and glands. Interneurons constitute by far the largest number of neurons in the brain. They can be divided into two groups. Projection, or relay interneurons (also termed Golgi type I cells) relay information over long distances, from one brain region to another, and accordingly have very long axons. Local interneurons (also termed Golgi type II cells) process information within specific subregions of the brain and have relatively short axons (Kandel, *et al.*, 1991).

Neurons are specially constructed to facilitate the conduction and processing of information. A typical neuron has four morphologically defined regions: (i) the cell body, or soma, consisting of the nucleus and perikaryon, (ii) dendrites, (iii) an axon, and (iv) presynaptic terminals. The axon and dendrites are processes that extend from the cell body. The dendrites typically branch out in a tree-like fashion and serve as the main input of information from other nerve cells. The axon is a tubular process ($\sim$0.2 to 20 μm in diameter) that can ramify and extend for up to one meter in humans. It carries, sometimes over great distances, the all-or-none electrical signals initiated in the cell body (action potentials) to other neurons or effector organs. The branches of the axon from a single neuron may form synapses with 1000 or more other neurons, while the dendrites and cell body of a single neuron (*e.g.*, a Purkinje cell of the cerebellum) may receive contacts from over 150,000 other cells. Near its end, the axon divides into fine branches with specialized endings termed presynaptic terminals.

The presynaptic terminals are the sites at which the neuron transmits its information to another cell (the postsynaptic cell). The postsynaptic cell may be another neuron, a muscle or a secretory (gland) cell. The point of contact is termed the synapse. In cases where the

postsynaptic cell is another neuron, presynaptic terminals typically terminate near the postsynaptic neuron's dendrites, although they may terminate at the cell body or at the initial segment of the postsynaptic axon. Mammalian CNS synapses are typically chemical in nature -- that is, they function by the presynaptic releasing a chemical neurotransmitter into the synaptic cleft. The neurotransmitter activates receptors in the postsynaptic cell membrane, which initiate a corresponding electrical or chemical (*i.e.*, calcium) signal in the postsynaptic cell.

Neurotransmitters used in the brain include glutamate and glutamate-like compounds, GABA, acetylcholine, catecholamines. Most neurons utilize a single neurotransmitter to communicate with other neurons or effector organs. Approximately 70% of the neurons in the human brain are glutamatergic, and ~30% are GABAergic. Less than one percent of the neurons use acetylcholine or catecholamines as transmitters (Peters, *et al.*, 1991).

The CNS neurons that communicate signals from the retina of the eye to the brain are termed retinal ganglion cells (RGCs). Retinal ganglion cells are generated in the rat embryonic retina approximately between E13 and E18 (reviewed in Jacobson, 1991). In rat, nearly all RGCs innervate the optic tectum (Linden and Perry, 1983). Their axons reach the tectum between E18 to P2 and establish a mature pattern of topographically correct tectal connections by about P8 (Simon and O'Leary, 1992). In humans, the retinal ganglion cells are typically generated between approximately E40 and E50.

2. Glia. Nerve cell bodies and axons are surrounded by glial cells. There are 10-50 times more glia than neurons in the central nervous system of vertebrates, and while they do not conduct or process information, they are essential for maintenance of normal function of the brain.

Glial cells of the vertebrate nervous system may be classified into two groups - microglia and macroglia. Microglia are phagocytes mobilized after injury, infection or disease. They are derived from macrophages, and are unrelated (physiologically and embryologically) to the cells of the central nervous system. Accordingly, the discussion herein is concerned with macroglia. Macroglia include three predominant types, two of which (oligodendrocytes and astrocytes) are present in the central nervous system (Kandel, *et al.*, 1991).

Oligodendrocytes are relatively small cells that form myelin sheaths around axons of the CNS by wrapping their processes concentrically around an axon in a tight spiral. A single oligodendrocyte typically envelopes several different axons (average of 15).

Astrocytes typically have irregularly-shaped bodies and relatively long processes. Some astrocytes serve as supporting cells for neurons, providing structure to the brain and separating groups of neurons from one another. Others contact endothelial cells of blood vessels in the brain, stimulating the endothelial cells to form tight junctions, which form the "blood-brain

barrier". Still others are involved in absorbing excess extracellular potassium or neurotransmitters released during episodes of high neuronal activity.

Optic nerve astrocytes may be further classified into type I and type II astrocytes. Type I astrocytes are the main type of white matter astrocytes *in vivo*. Their processes contact nodes of Ranvier and blood vessels. Type II astrocytes are commonly found *in vitro* in CNS cultures, but are not normally observed *in vivo*. It is possible that type II astrocytes are induced following injury.

As is described in greater detail below, glial cells can also serve to secrete certain survival factors required to prevent the neurons from undergoing apoptosis. Whereas both astrocytes and Schwann cells have previously been found to secrete a variety of neurotrophic factors, including brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), and insulin-like growth factor 1 (IGF-1), there has heretofore been no evidence to suggest that oligodendrocytes, the myelinating cells of the CNS, secrete neurotrophic factors.

IV. Conditions Effective to Promote Neuronal Survival *in vitro*

The teachings provided herein enable one of skill in the art to maintain cultures of pure CNS neuronal cells under defined conditions for extended periods of time. These conditions include growing or maintaining the cells under conditions effective to stimulate cAMP-dependent protein kinase in the cells and providing at least one trophic growth factor. As will be discussed more fully below, the inclusion of one growth factor effectively promotes short-term (3 day) survival of approximately >30% of such CNS neuronal cells. Inclusion of more than one factor, which or may not be selected from a different "class" than the first factor (see below), increases the rate of survival relative to that seen with a single factor. Similarly, adding a third factor, which again may or may not be from still another "class", extends the survival beyond that seen with two factors.

Results of experiments detailed in Example 1 illustrate these relationships. Individual growth factors were applied either in isolation (one factor per experiment) or in combination (two or more factors per experiment) to cultures of purified rat retinal ganglion cells. The factors were applied with or without forskolin, a compound which activates adenylate cyclase and thereby increase intracellular levels of cAMP.

The results of the studies are summarized in Table 2. High concentrations of single peptide growth factors applied in isolation in the absence of agents effective to elevate intracellular concentrations of cAMP (such as forskolin) did not promote the survival of more than about 2% of cells. In contrast, when the factors were applied in the presence of forskolin, the survival of the cells was increased approximately 10-fold relative to the action of the factor in the absence of forskolin. Other cAMP elevating agents, as well as cAMP analogues which activate cAMP-

dependent protein kinase (*e.g.*, chlorophenylthio-cAMP (CPTcAMP)), may be used in place of forskolin with similar effect.

Also detailed in Example 1 are experiments measuring the dose-response curves of the survival-promoting ability of BDNF and CNTF (in the presence of forskolin and insulin) on purified RGCs. The results, shown in Figure 2A, show that 2 ng/ml BDNF, and 0.2 ng/ml CNTF promoted half-maximal survival.

The above results indicate that the short-term survival (survival at 3 days of culture) of RGCs was promoted by the application of single trophic factors in combination with cAMP-elevating agents or cAMP analogs (acting, as described below, to stimulate cAMP-dependent protein kinase). The addition of factors effective to stimulate cAMP-dependent protein kinase elevated the survival at 3 days from <3% to between 15 and 40%. When BDNF, NT-4/5, CNTF or LIF were used, the survival was elevated to approximately 30%.

As stated above, the factors used may be grouped into classes based on structural features and functional characteristics. The first class, termed insulin-like growth factors, includes insulin, insulin-like growth factor 1 (IGF-1; previously referred to as somatomedin C) and insulin-like growth factor 2 (IGF-2; previously referred to as Multiplication Stimulating Activity). IGF-1 and IGF-2 share about 76% sequence identity with each other and about 50% sequence identity with proinsulin. IGF-1 is thought to be an autocrine regulator of skeletal growth and whole-body protein metabolism, while IGF-2 is thought to be involved in fetal development (LeRoith and Roberts, 1993; Lowe, 1991). Both IGF-1 and IGF-2 are nonglycosylated single-chain peptides, 70 and 67 amino acid residues in length, respectively.

The second class of growth factors, termed neurotrophins, includes brain-derived neurotrophic factor (BDNF) and neurotrophin 4/5 (NT-4/5). BDNF is a small ($\sim$12 kDa), basic protein originally purified from mammalian brain (Barde, *et al.*, 1982). NT-4/5 was originally isolated from *Xenopus* and viper DNA (Halbook, *et al.*, 1991). The equivalent mammalian gene, also known as NT-5, was subsequently isolated from rat and human DNA (Halbook, *et al.*, 1991; Ip, *et al.*, 1992). NT-4/5 shares a 50-60% amino acid homology with other members of the neurotrophin family

The third class of growth factors, termed CNTF-like cytokines, includes ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF) and interleukin-6 (IL-6). Oncostatin M (OSM) and interleukin-11 (IL-11) may also be considered to be CNTF-like cytokines. CNTF was initially purified from chick eye, and has been demonstrated to have survival-promoting activity on chick ciliary, dorsal root sensory and sympathetic ganglia (Barbin, *et al.*, 1984). Human IL-6 cDNA encodes a 212 amino acid precursor that is cleaved to produce a mature protein (minus the N-terminal signal peptide) of 184 amino acids and a molecular weight of 21 kDa. IL-6 is expressed by a variety of cells, including astrocytes, and has been shown to

stimulate growth in a number of systems. LIF is pleiotropic cytokine initially identified as a factor that could inhibit proliferation and induce macrophage differentiation in the murine myeloid leukemic cell line M1 (Gearing, *et al.*, 1987).

Other factors which are similar (structurally and/or in terms of biological activity) to factors in one of the classes discussed above may evaluated for survival-promoting activity as detailed herein, and if effective, be employed in place of the factors of that class recited above in the practice of the present invention.

Combinations of two or three factors from within a class did not give a significantly better survival than the individual factors alone. In contrast, however, experiments detailed herein show that a combination of factors from different classes produced additive effects on survival.

Exemplary results are summarized in Figure 2B, which shows the effects of combining survival factors on short term (3 day) retinal ganglion cell survival. All factors were used at plateau concentrations (concentrations providing the maximum percent survival in assays such as shown in Fig. 2A). Insulin plus BDNF, for example, was better than insulin or BDNF alone, while insulin plus BDNF and CNTF was better than any two of these alone. The majority of the cells could be saved for at least 3 days by the combination of forskolin, insulin, BDNF, CNTF and growth on a merosin-coated surface.

In the presence of two of the three factors, under conditions effective to stimulate cAMP-dependent protein kinase (*e.g.*, 5 μM forskolin), at least about 30% of the cell could survive for up to about 7 days. These results suggest that growth factors from the three classes of growth factors described above do not promote the survival of different subsets of cells, but act synergistically on individual cells to promote long-term survival.

By employing a combination of three trophic factors, one from each of the classes recited above, in conjunction with PKA-activating conditions, a pure serum-free culture of healthy CNS neurons may be maintained for at least 28 days. Further, the rate of change of survival as a function of time in these studies suggests that the majority of cells may be able to survive under such conditions for up to 60 days, with at least 30% surviving upwards of several months. Exemplary results of experiments to this effect are detailed in Example 1C and shown in Fig. 3.

Taken together, the above results indicate that (i) single peptide growth factors (e.g., BDNF, NT-4/5, CNTF or LIF), applied individually, can promote survival for up to about one week of ~30% of purified CNS neuronal cells grown under conditions effective to stimulate cAMP-dependent protein kinases in those cells (*e.g.*, increased levels of intracellular cAMP), (ii) combination of peptide trophic factors selected from at least two of the three classes described above can promote survival of ~30% or more of such cells for up to about 7 days, and (iii) three trophic factors, one each from the three classes defined above, can promote survival of such cells for over a month.

It will be appreciated that the trophic factors employed in the present experiments are secreted by cells that (*in situ*) normally contact the CNS neurons being cultured. Such contacting cells include glial cells (astrocytes and oligodendrocytes) as well as cells with which the neurons forms synaptic connections (presynaptic cells and postsynaptic cells). Examples of effective survival factors secreted by neighboring cells included BDNF (made by the tectum), CNTF or LIF (made by optic nerve astrocytes and tectum; Stockli, *et al.*, 1991; Yamamori, 1991; Patterson and Fann, 1992), and IGF-1 (made by astrocytes; Ballotti, *et al.*, 1987; Hannson, *et al.*, 1989; Rotwein, *et al.*, 1988; Baron-van Evercooren, *et al.*, 1991). The results summarized above suggest that long-term survival of CNS neurons, such as RGCs, depends on a collaboration of astrocyte, oligodendrocyte and target-derived factors, as well as stimulation of cAMP-dependent protein kinases in the cells (*e.g.*, by elevation of intracellular cAMP, accomplished, *e.g.*, by forskolin).

In addition, the data discussed above suggest that the requirement for multiple trophic factors for long-term survival does not reflect the presence of multiple subsets of cells, each with different trophic factor sensitivities, but rather, that multiple factors act collaboratively on individual RGCs to promote their long-term survival.

Surviving neurons cultured under conditions described herein typically possess the same types of cell-specific markers and physiological properties as they would *in vivo*. A number of neuronal markers are available to assess this. For example, the marker Thy-1, which was originally found on thymocytes (Williams and Gagnon, 1982), is expressed on mature glutamatergic projection neurons, such as retinal ganglion cells and cerebral cortical glutamatergic projection neurons, and may be used to purify such cells (as detailed below) as well as to identify them from other cell types, and to assess their general conditions (*i.e.*, healthy, differentiated glutamatergic projection neurons express Thy-1). Thy-1 is a major brain glycoprotein found on the surface of many neurons, and is a member of the Ig superfamily.

Other neuronal marker are known to those skilled in the art. Two such markers, which are particularly useful for assaying the health and differentiated state of neurons, include MAP-2 and tau. MAP-2 refers to microtubule-associated protein 2, an intracellular cytoskeletal protein that is expressed in the dendrites of a wide variety of CNS neurons, but is not expressed in non-neuronal cells (Kosik and Finch, 1987). When neurons in culture lose viability, they typically retract their dendrites and stop expressing MAP-2. Tau is expressed in the axons of a wide variety of neurons, but like MAP-2, is not expressed in non-neuronal cells. Tau is also an intracellular cytoskeletal protein, and may be used as a marker for healthy CNS neurons.

Examples of the use of anti-MAP-2 and anti-tau antibodies to stain CNS neurons are presented in Example 7. Purified retinal ganglion cells grown in serum-free MBS cultures containing BDNF, CNTF, high insulin, and forskolin were fixed and stained, at culture day 7,

with an anti-MAP-2 monoclonal antibody (Fig. 11A) or an anti-tau monoclonal antibody (Fig. 11B) as described in Materials and Methods, below. The primary antibodies were detected with an FITC-conjugated anti-mouse IgG antibody. As can be appreciated from the photomicrographs, the cells clearly express the neuronal markers MAP-2 and tau.

Several specific markers exists for glial cells as well. For example, optic nerve type-1 astrocytes express an unknown cell membrane protein termed RAN-2 (rat neural antigen 2; Bartlett, *et al.*, 1981), oligodendrocyte precursor cells express the ganglioside A2B5 (Eisenbarth, *et al.*, 1979), and mature oligodendrocytes express galactocerebroside glycolipid (GC; Ranscht, *et al.*, 1982). Antibodies directed against these cell surface markers may be used to purify the respective cell populations, as described below, and to identify the cells using, for example, immunohistochemistry.

In addition to immunohistochemical methods for assessing cell characteristics, electrophysiological approaches may also be employed. For example, patch clamp recording may be used to characterize the distribution and types of ion channels present in the cell membrane, or to record the membrane response to depolarizing stimuli, as detailed, *e.g.*, in Example 7.

Methods of the present invention may be employed to culture both embryonic (prenatal) CNS neurons as well as postnatal CNS neurons. When cultured in a serum-free medium in the absence of feeder cells or conditioned medium, both types of cells need trophic growth factors as well as conditions effective to stimulate intracellular cAMP-dependent protein kinases (*e.g.*, elevated intracellular cAMP). The specific types of trophic factors required may differ between the two cell types, however. In particular, neurons that have not yet contacted their targets at the time they are removed from an organism for culturing typically require trophic factors produced by the target cells. Further, such cells may have altered requirements (relative to postnatal cells) for other trophic factors, such as factors of the CNTF-like cytokine family.

For example, experiments performed in support of the present invention and detailed in Example 5 demonstrate that survival of cultured embryonic day 18 (E18) RGCs, most of which have not yet innervated the tectum, is promoted ($\sim$30% of cells surviving at 3 days) by insulin and BDNF (in the presence of forskolin), but is not significantly affected by CNTF or LIF. Further, unlike postnatal RGCs, the E18 cells do not respond to bFGF, TGF-α, RGC paracrine factor or oligodendrocyte-derived neurotrophic factor.

Additional experiments performed in support of the present invention (detailed in Example 5) also demonstrate, however, that a high degree of survival ($>$75% at 3 days) may be obtained if the cells are cultured in the presence of medium conditioned by purified tectal cells (Fig. 7). The survival of the E18 RGCs was enhanced nearly 5-fold (relative to no treatment) by soluble tectal factors. The results indicate that the tectum secretes a significant survival activity which, according to additional experiments performed in support of the present invention, was not

mimicked by known neurotrophins, including nerve growth factor (NGF), BDNF, neurotrophin-3 (NT-3), and NT4/5.

Photomicrographs of the purified E18 retinal ganglion cells in the absence and presence of tectal medium are shown in Figs. 8A and 8B, respectively. Whereas the majority of cells grown in the absence of the tectal medium died by three days of culture, the majority of those grown in the presence of the tectal conditioned medium thrived and extended processes.

In cases such as described above, where the CNS neurons are isolated at a developmental stage at which they had not yet reached their intended targets, trophic factors that are synthesized by those targets may be employed in the practice of the present invention to promote the survival of cultures of such neurons, and to promote survival and regeneration of damaged CNS neurons *in vivo*. Such target-derived factors may be used in addition to factors discussed above, or in certain cases, in place of a representative factor from one of the three classes discussed above. Experiments detailed below demonstrate how one of skill in the art may determine the minimal required combination of factors for promoting survival.

V. Activation of cAMP-Dependent Protein Kinase

According to the present invention, an important aspect of promoting the survival of pure cultures of CNS neurons under chemically-defined (*e.g.*, serum-free) conditions is promotion of conditions effective to activate of cAMP-dependent protein kinase (protein kinase A; PKA) in the cells. Such activation is typically accomplished by elevation of intracellular cAMP levels or the application of a cAMP analogue effective to activate cAMP-dependent protein kinase. While not committing to any underlying mechanism, it is a discovery of the present invention that conditions that activate cAMP-dependent protein kinases, such as MAP kinase (erk1), also promote survival of neuronal cells *in vitro* and *in vivo*.

Experiments detailed herein demonstrate a number of ways by which cAMP-dependent protein kinase may be activated to levels that promote survival of CNS neurons (when appropriate growth factors are used). The most commonly-used of these methods was the application of forskolin. Forskolin activates adenylate cyclase, the enzyme that synthesizes cAMP, thereby directly increasing intracellular levels of cAMP. Forskolin was employed in RGC experiments detailed in Examples 1-7 and 9, and in cortical neuron experiments detailed in Example 8. The addition of forskolin to the culture medium generally resulted in a 6-10 fold increase in the percent survival relative to no forskolin.

The experiments described in Example 8 (results in Fig. 13) show typical results of forskolin. Thy-1 positive cerebral cortical neurons were plated in MBS medium containing BDNF, CNTF, insulin and forskolin, individually or in combination as indicated, and their survival plotted along the y-axis.

The results, shown in Fig. 13, demonstrate that single factors applied individually (BDNF, CNTF) resulted in less than about 5% survival. Two factors applied together resulted in approximately 10% survival, and three factor about 15% survival. When forskolin was present in the culture medium, however, the number of cells surviving under each set of conditions was increased by about 6 to 10 -fold.

The results indicate that (i) individual growth factors in the presence of cAMP-elevating agents promote survival and maintenance of CNS neurons, (ii) multiple factors promote a higher degree of survival than individual factors, with two factors being better than one, and three being better than two, and (iii) the presence of forskolin dramatically increases the survival with any given growth factor combination.

In addition to forskolin application, a number of other methods may be used to stimulate cAMP-dependent protein kinase. For example, experiments described herein demonstrate that depolarizing the cells in the presence of physiological levels (about 200 μM to 10 mM) of extracellular calcium is effective to confer the same potentiation on survival caused by forskolin. The depolarization may be achieved by increasing the extracellular potassium concentration, as illustrated in Example 6 and Fig. 9A, by activating glutamate receptors using a cocktail of glutamate receptor agonists, as illustrated in Example 6, Fig. 9B, or by other means, such as electrical stimulation. The depolarizing stimuli appear to converge on the same molecular mechanism as forskolin activation, since both are blocked by the application of Rp-cAMP, a cAMP-dependent protein kinase inhibitor (Figs. 9A, 9B).

Analogs of cAMP, particularly membrane-permeant analogs such as chlorophenylthio-cAMP (CPTcAMP), are also effective at activating PKA, and may be used in combination with growth factors in methods of the present invention.

VI. Additional Culture Conditions Affecting Survival

Cell culture techniques which have been employed in the past by skilled artisans to enhance growth and differentiation of cells in serum-containing media may also be employed with methods of the present invention to further enhance the growth and differentiation of purified CNS neurons grown under defined conditions.

For example, appropriate choice of substrate is an important aspect of cell survival. Experiments performed in support of the present invention suggest that optimal CNS neuronal survival is obtained when the cells are cultured on a substrate that is coated with matrix proteins or is otherwise modified to enhance cell growth. Specifically, substrates containing the matrix proteins laminin and/or merosin promote CNS neuronal survival and neurite outgrowth when the cells are grown according to the methods of the present invention (*i.e.*, grown under conditions

effective to stimulate cAMP-dependent protein kinase in the presence of one or more growth factors).

Similarly, appropriate choice of the defined components added to the serum-free media described herein is important for optimal neuronal cell survival. For example, the components identified in Table 1, particularly progesterone, tri-iodothyronine and thyroxine, are important contributors to CNS neuronal cell survival.

The survival of CNS neuronal cells under defined conditions may also be also affected by soluble factor(s) released by the neuronal cells themselves. For example, it is known that for certain cultures that survival time can be increased by increasing the density at which the cells comprising the cultures are plated. This density effect on survival is presumably due to trophic factors, secreted by the cultured cells, that need to be present above a threshold concentration for optimal cell survival. According to the teachings herein, such factors may be added to neuronal cultures of the present invention to further promote survival of the cells, particularly if the cells are plated at low densities.

For example, experiments performed in support of the present invention and detailed in Example 2 indicate that RGC survive better when plated at a high density rather than at low density, and that the survival rate of cells plated on a coverslip at low density can be increased by incubating the coverslip above a conditioning layer of high-density RGCs. Exemplary results of these experiments are shown in Figures 4A and 4B for 3 day cultures (Fig. 4A) and 7 day cultures (Fig. 4B). Additional experiments (Fig. 4B) demonstrated that at least a part of the effect of cell density on survival was due to basic fibroblast growth factor (bFGF), which is expressed by retinal ganglion cells *in vivo* (Elde, *et al.*, 1991; Connolly, *et al.*, 1992).

The support on which the cells are plated can also have a significant effect on cell survival. In particular, it will be noted that the survival of CNS neurons plated on glass is significantly worse than the survival of the same cells plated on plastic (e.g., tissue culture plastic), other factors being equal. This difference is exemplified in the difference in survival rates shown in Figure 3 (plated on plastic) vs the survival rates indicated in Table 3 (plated on glass). For example, the survival rate for RGCs in Table 3 at 3 days in medium containing forskolin, CNTF, BDNF, and insulin was 55%, whereas the survival rate for the same cells grown in the same medium, but plated on plastic, was 80% at 3 days (Figure 3).

VII. Promotion of Survival of CNS Cells *in vivo*

As mentioned above, many of the observations made in the *in vitro* experiments described above are directly transferrable to *in vivo* situations. Experiments carried out in support of the present invention confirm this observation.

Methods for measuring *in vivo* correlates were initially tested in culture. For example, in order to visualize elevation of cAMP levels in intact cells, immunofluorescence studies using anti-cAMP antibodies were established, according to standard methods known in the art, and further described in Materials and Methods and Example 14, below. Figures 14A-14C confirm that forskolin treatment results in concentration-dependent increases in intracellular cAMP, as measured using this method. Parallel results were observed using a cAMP radioimmunoassay in extracts prepared from the cells.

It was further observed that in order to visualize cAMP elevation *in vivo*, it is useful to include in the treatment method a phosphodiesterase inhibitor such as IBMX. While such a compound may also be used in *in vivo* treatment methods, it should be emphasized that the treatment method may persist in the absence of such a compound. (But see discussion of Figure 20, below, for evidence that such a compound further enhances neuronal survival).

Further experiments carried out in support of the present invention indicated that depolarizing conditions were effective to raise intracellular cAMP levels to a sufficient degree to promote survival of neurons. As shown in Figure 15, depolarizing conditions, exemplified by exposure to glutamate or high KCl treatments resulted in increased intracellular concentrations of cAMP, as assessed by immunofluorescence (anti-cAMP antibody; Example 14)

In further studies supporting the present invention, it was found that in cultures of purified retinal ganglion cells, responsiveness to growth factors can be followed by quantifying the percentage of cells that translocate MAP kinase (erk1) into their nuclei following stimulation. Translocation of MAP kinase is stimulated by cAMP elevation in the cytosol. In RGC cultures containing either growth factors alone or forskolin alone, only about 10% of cells exhibit nuclear erk1 (MAP kinase) immunostaining. When the RGCs are stimulated by growth factors together with forskolin, nuclear translocation of erk1 occurs in about 80% of cells. These results are illustrated in Figure 16, where the number of cells with translocated MAP kinase is indicated on the abscissa as a function of time. Lines labeled "cAMP" are plots obtained from cells exposed to 5 μM forskolin. As shown, the combination of BDNF and forskolin resulted in a marked enhancement of MAP kinase translocation to the nuclei of the cells.

As indicated above, the foregoing *in vitro* experiments were confirmed *in vivo*. An exemplary *in vivo* model system for neuronal survival in the CNS is the retina, the physiology and anatomy of which were discussed in Section III, above. The results from this system are directly applicable to other parts of the CNS, such as the spinal cord or the brain. While optimization of results in other regions may depend on the biochemical and pharmacological attributes of particular region (for purposes of optimizing particular mix of growth factors), as well as the volume of the region (for purposes of determining dosage), it is appreciated that with the guidance

of the present specification, such optimization will be well within the grasp of the skilled practitioner.

As described in Example 16, exemplary retinal experiments were carried out by injecting substances directly into the vitreous fluid of the rat eye, or by excising whole rat retina by dissecting the retinas into a dish with basal medium. Figure 17 shows the results of experiments in which whole retinas were exposed to 0.1 mM IBMX alone or IBMX with forskolin (5 μM), then tested for cAMP levels using anti-cAMP antibodies, as described above. In prior experiments (not shown) lower levels of activation were observed when forskolin was used alone. Here, as shown the combination of IBMX+forskolin provided measurable activation of adenylate cyclase activity in the whole preparations, as evidenced by increased levels of cAMP.

As discussed above, translocation of MAP kinase in response to cAMP can be correlated with conditions which promote CNS neuronal cell survival *in vitro*. Experiments described in Examples 17 and 18 provide a similar correlation for CNS neurons *in vivo*.

Figure 18 shows the results of experiments in which whole retinas treated with a cocktail of growth factors (BDNF and CTNF), forskolin and IBMX (0.1 mM) showed enhanced nuclear translocation of MAP kinase than control retinas. Further quantitation of this phenomenon is shown in Figure 19, where "Factors" indicates retinas treated with BDNF and CTNF (50 μg/ml each), and "cAMP" indicates retinas treated with 5 μM forskolin in the presence of IBMX (0.1 mM). In this experiment, blockers of depolarization (tetrodotoxin (TTX), curare, and kynurenic acid (Kyn) were able to reverse the translocation stimulatory effects of the growth factor/forskolin cocktail. These experiments further the correlation between cAMP activation and MAP kinase translocation.

Further studies were carried out in intact retinas in rats. Here, as detailed in Example 18, P8 retinal ganglion cells were retrogradely labelled *in vivo* using fluorogold. The retinal ganglia were then transected retroorbitally to produce "degenerating conditions", as evidenced by almost total retinal ganglion cell death after 3 days in untreated retinas ("Sham", Figure 20). In contrasts, eyes that were subjected to intraocular injection of a cocktail containing BDNF, CNTF and IGF-1 ("factors" in Figure 20), a cocktail of the same factors + forskolin, or a cocktail of factors, forskolin + IBMX, showed enhanced survival. Of particular relevance to the present invention, the treatment method that ensured continuously high cAMP levels throughout the degeneration period (presence of IBMX) resulted in significantly higher survival rates than factors alone or factors + forskolin. Figure 21 shows computer-generated images of fluorogold-labeled retinal ganglion cells in the absence (panel A) or presence (panels B and C) of degenerating conditions (axotomy ("cut"), as described above), and shows that the presence of elevated cAMP and factors resulted in increased numbers of surviving cells (panel C) compared to the untreated condition (panel B).

VIII. Common Characteristics of CNS cells

The experiments detailed herein have employed two exemplary types of CNS neurons, retinal ganglion cells and cortical projection neurons, to demonstrate survival and growth factor requirements of the present invention which may be applied to the culture of CNS neuronal cells in general. Both retinal ganglion cells and cortical projection neurons can be classified as glutamatergic projection neurons, a broad category which includes approximately 70% of the neurons in the human brain. The fact that, according to the teachings herein, these two cell types have essentially identical growth and survival requirements, despite their different presynaptic inputs, locations and target cells in the CNS, suggests that the conditions detailed herein for their growth and survival may be applied to other types of CNS neurons.

Supporting this teaching is the fact that the retinal ganglion cells and the cortical projection neurons share, along with essentially all the other neurons of the central nervous system, intimate contact with astrocytes and oligodendrocytes, the two basic types of glial cells present in the central nervous system. Experiments exemplified below demonstrate that under conditions described herein (*i.e.*, conditions effective to stimulate or activate cAMP-dependent protein kinases), specific factors secreted by astrocytes and oligodendrocytes (*e.g.*, CNTF, IGF-1), are effective at promoting the survival of the CNS neurons that the glia contact, irrespective of other cells (*e.g.*, presynaptic cells or postsynaptic target cells) which may contact those neurons.

The experiments also compared the specific growth factor requirements of pure cultures of one CNS neuronal cell type (retinal ganglion cells) at two developmental stages (isolated before target innervation and after target innervation). The results of these studies indicate that while CNS neuronal cell requirements for specific factors may differ somewhat as a function of the cells's developmental stage at time of isolation (*i.e.*, whether or not the cell had innervated its target), the basic teachings herein regarding CNS neuronal cell survival (*i.e.*, PKA activation w/trophic factors) apply to all CNS cells. Further, it will be appreciated that in spite of some variation in the minimal set of necessary factors (minimal complement) to promote optimal survival, the inclusion of, for example, one factor from each of the 3 factor classes described above, effectively promotes survival of the cultures. Accordingly, it may not be necessary to determine in every case what the exact minimal complement of factors is -- survival of the cells may be promoted using three different factors as described above, and in cases of cells that had not yet innervated their targets, may be further promoted by the addition of a factor or factors derived from those targets.

IX. Utility of PKA Stimulation and Growth Factors in Treatment of CNS Injury and Regeneration

It will be understood that the survival and growth conditions determined from the *in vitro* culture experiments described herein may be applied to promote the survival, growth and regeneration of injured CNS neurons *in vivo*. Following a CNS neuronal injury, such as injury caused by acute trauma, ischemia/reperfusion, neurodegenerative disease, and the like, the injured neurons typically retract their axons and commit apoptosis. Presynaptic cells which terminate on the injured neuron withdraw their inputs, and glial cells contacting the injured neuron die.

Injury resulting in interruption of axons in the adult mammalian CNS leads to a persistent neurological defect, such as paralysis or blindness, because of the failure of the CNS to repair itself. In particular, injury to RGC axons leads to blindness because of death and permanent loss of the RGCs and their axons (Berkelaar, *et al.*, 1994).

Previous studies have shown that when the optic nerve was transected near the eye and replaced with a sciatic nerve graft (Bray, *et al.*, 1991), at least some RGCs could both survive and successfully regenerate their axons, ultimately to form functional synaptic connections in the tectum. However, the sciatic nerve grafts allowed only a small percentage of RGCs to survive and regenerate (about 5-10%), and it remains unclear as to why the sciatic nerve environment was better than the optic nerve environment for promoting survival. Heretofore, it has not been possible to regenerate CNS axons in the absence of PNS tissue with any useful degree of success.

A. Delivery of Growth Factors to the Injured Cells.

Growth factors may be delivered to CNS neurons *in vivo* using a number of methods. For example, local application to a site of acute injury may be via a direct injection to the affected site (*e.g.*, intraocular injection for promoting retinal ganglion cell survival following optic nerve injury or injection into the cerebrospinal fluid for promoting survival of cells with damaged axons following spinal cord injury). The injections may be administered periodically until the injury has been effectively treated. The factors may also be delivered systemically (e.g., intravenous injection), particularly in cases where the blood-brain barrier is compromised or broken-down, such as may occur in trauma or inflammatory disease (e.g., multiple sclerosis).

Preferably, in accordance with the present invention, survival of CNS neuronal cells will be promoted by exposing a target cell region to (i) at least one growth factor selected from the group consisting of insulin-like growth factors, neurotrophins, and CNTF-like cytokines, as discussed above, and (ii) conditions effective to produce elevated levels of cyclic adenosine monophosphate (cAMP) in the cells, as evidenced by ability to stimulate a cAMP-dependent protein kinase in said cells. Exemplary conditions and means for assessing elevation of cAMP and activation of protein kinase were discussed in Section VII, above. As described above, it

will be appreciated that optimization of conditions may differ from cell-type to cell-type; however, as evidenced by the cortical cell experiments discussed above, the general principles are applicable to cells from varied regions of the CNS.

Means for activating adenylate cyclase and/or protein kinases were discussed above. They include, but are not limited to exposing the cells to activators of adenylate cyclase, such as forskolin or cholera toxin (or fragments thereof), adding membrane permeable cAMP analogs to the cells such as are known in the art, or depolarizing the cells, such as by electrical probe stimulation or exposure to glutamate agonists, according to methods well known in the art. Promotion of cell survival may be further enhanced by inclusion in a treatment cocktail of an inhibitor of phosphodiesterase, such as IBMX, exemplified herein, theophylline, caffeine, or the like as discussed below. Preferably, the foregoing cocktail will be included in a suitable pharmaceutical excipient, of which sterile saline is an example. Such an excipient may be augmented with stabilizing agents, thickening agents, or other additives known in the art to promote chemical stability and localization of the mixture to a particular region, particularly since it may be preferable to inject the cocktail into a discrete retion, such as the vitreal fluid, as exemplified herein, particular regions of the spinal cord or brain, and the like. Practitioners will understand that the guidelines presented herein for selection of a particular stimulatory cocktail, including concentrations and components thereof, will be augmented by practical knowledge concerning the pharmacodynamics and pharmacokinetics of a particular application site and toxicology of such substances, such as may be known in the art or empirically determined.

As an alternative mode of application, cells secreting recombinant factors may also be administered to deliver selected factors following, *e.g.*, an acute CNS injury. Such cells can effectively secrete the desired factors for over two weeks, facilitating healing of the injured tissue. A cell-mediated delivery approach such as described above has been used successfully to deliver proteins as large as antibodies throughout the CNS. For example, Schnell and Schwab (1990) showed that when antibody-secreting hybridoma cells are injected into postnatal rat brain, the antibodies are secreted into the spinal fluid and delivered throughout the brain, optic nerve and retina.

Peptide trophic factors can be delivered using similar approaches by injecting cells that had been transiently or stably transfected with a vector containing peptide trophic factor DNA sequences in a manner effective to cause the cells to secrete the recombinant trophic factor into the subarachnoid space (Barres, *et al.*, 1992, 1993, 1993a, 1994; Barres and Raff, 1993; Example 13). Such cells sink to the basilar meninges overlying the optic chiasm and continuously deliver trophic factors at high levels into the optic nerve for at least two weeks.

This approach is simple, has a high success rate, and causes no apparent discomfort to the postnatal rat pups in which it was employed. Using this approach, experiments performed in

support of the present invention have shown that delivery of the exogenous survival factors CNTF, IGF-1, NT-3 or PDGF into the developing optic nerve significantly decreases the normal cell death of newly-formed oligodendrocytes. The transfected cells can deliver secreted factor for at least 10 to 14 days, ample time to allow significant regeneration to occur (axon regeneration should occur at the rate of slow axonal transport, which is 1 mm per day; the postnatal optic nerve is less than 10 mm long).

The factors may also be introduced using gene therapy approaches. Such modes of administration may be particularly suitable for the delivery of factors in cases of chronic neurological disorders, such as ALS, Alzheimer's disease or multiple sclerosis. DNA sequences encoding the desired factors are cloned into a suitable expression vector effective to express the factor in selected host cells. For example, RGCs may be directly targeted with a replication-deficient herpes virus vector containing DNA sequences encoding a desired growth factor. Herpes virus vectors (Breakefield and DeLuca; Freese, *et al.*) are particularly well suited for the delivery and expression of foreign DNA in cells of the CNS, since they can efficiently infect mature, postmitotic neurons. Methods for manipulating the vectors and transfecting CNS cells are well known (see, *e.g.*, Kennedy and Steiner; Yung). A number of studies describe methods for transplanting genetically modified cells into different regions of the brain (Malin, *et al.*, 1989, 1992; Rossi and Sarver; Sullenger, *et al.*; Chatterjee, *et al.*; Hope, *et al.*). Studies utilizing direct injection of vectors into CNS tissue have also been performed (*e.g.*, Zhang, *et al.*).

Herpes virus vectors have additional advantages for CNS regeneration therapy. For example, failure of the upper motor neurons (pyramidal glutamatergic projection neurons that synapse onto the lower motor neurons in the spinal cord) to regenerate their axons is the main cause of paralysis after a spinal cord injury. Since herpes viruses can be retrogradely transported, the upper motorneurons may be targeted by local injection of an injured corticospinal tract. Herpes virus vectors capable of expressing appropriate growth factors can be administered in this way under conditions which stimulate PKA in the injured cells to promote regeneration and reduce or eliminate paralysis following spinal cord injury.

Alternatively, appropriate expression vectors may be used to transfect glial cells (*in vivo* or *in vitro*), such as astrocytes or oligodendrocytes, to produce cells capable of secreting a desired recombinant growth factor. Cells transfected with different factors may be used together in the methods of the present invention to deliver multiple factors to the same site. Cells transfected *in vitro* may be transplanted back into the region of the brain in need of treatment as described above. Transfected cells secrete the desired factors and promote survival of the degenerating neurons.

B. Stimulation of PKA in Injured Cells.

Protein kinase A may be stimulated *in vivo* by pharmacological approaches, genetic approaches or electrical stimulation. For example, the injured cells may be stimulated with a stimulating electrode (*e.g.*, an implanted electrode), or by administration of an agent effective to depolarize the injured cells (*e.g.*, a glutamate agonist, or preferably, a mixture of glutamate agonists). An exemplary implantable electrode suitable for electrical stimulation of retinal ganglion cells has been described (Roush). Electrodes suitable for the stimulation of other CNS regions, such as spinal cord, are also known.

Alternatively, PKA may be stimulated by pharmacologically elevating cAMP levels in the injured neurons, such as by injecting, for example, forskolin, CPTcAMP or 8-Bromo-cAMP periodically to the affected site. Methylxanthines, such as caffeine and theophylline, are clinically-approved drugs that may also be used to elevate intracellular cAMP concentrations.

X. Utility of Defined CNS Cultures

Previously, cultures of CNS cells have typically used serum or a layer of feeder cells to provide necessary growth factors and undefined "growth promoting" signals. In certain applications, however, it would be highly desirable to avoid the use of such undefined components and employ the methods detailed herein.

For example, methods of the present invention may be used to grow pure cultures of CNS neurons under defined conditions for use in screening of therapeutic substances. Such screens should be as uniform as possible to allow standardization of test results. It is well known in the art, however, that the physiological characteristics and/or patterns of gene expression in cultured cells grown in the presence of serum often differ depending on the lot of serum used. Such inconsistencies may result in irreproducible or erroneous screening results, leading significant downtime and ensuing economic losses as the source of the variability in cell is traced down.

In cases where neuronal cells are being maintained in culture for subsequent transplantation into individuals in cell-based therapy applications, such variability may result in ineffective or potentially dangerous cell formulations being administered to the recipient. Further, unknown or undefined contaminants (*e.g.*, trace antibiotics) present in the serum may be taken up by the cells and pose potential problems for the recipient. Examples of such applications cells used to treat patients with Parkinson's disease. Fetal CNS cultures such as those described above may be conveniently grown under defined conditions using methods of the present invention, and thus avoid the undesirable characteristics necessarily associated with cultures grown in serum and/or conditioned medium.

The retinal ganglion cell data exemplified herein are particularly relevant to humans and human conditions, since rat optic nerve has a structure that is very similar to that of humans

(Kennedy, *et al.*, 1986). All of the three main types of glial cells found in rat optic nerve are found in human optic nerve. Many of the antibody markers used to study rat optic nerve also identify human optic nerve glia. Thus, these experiments are directly relevant to understanding the function and pathophysiology of human glia.

Further, the *in vivo* aspect of the present invention has direct implications in the treatment of degenerative conditions of the CNS, such as acute injury to a portion of the central nervous system (trauma and/or ischemia) or a neurodegenerative disorder, such as Alzheimer's disease or amyotrophic lateral sclerosis. With particular relevance to the retinal data, the disorder may also be, for example, glaucoma or macular degeneration, or a demyelinating disorder, such as multiple sclerosis. ****

XI. Oligodendrocyte-Derived Neurotrophic Factor

Whereas both astrocytes and Schwann cells have previously been found to secrete a variety of neurotrophic factors including brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), and insulin-like growth factor 1 (IGF-1) (Stockli, *et al.*, 1991, Yamamori, 1991, Patterson and Fann, 1992, Ballotti, *et al.*, 1987, Hannson, *et al.*, 1989, Rotwein, *et al.*, 1988), there has heretofore been no evidence that oligodendrocytes, the myelinating cells of the CNS, secrete neurotrophic factors. Experiments performed in support of the present invention and detailed in Example 3, below, indicate that cultured oligodendrocytes can secrete a trypsin-sensitive, heat-sensitive factor that promotes the survival of CNS neurons cultured under defined conditions. The factor was identified by culturing purified P8 RGCs over a conditioning layer of highly purified postnatal optic nerve astrocytes (>99.5% pure), immature oligodendrocytes (oligodendrocyte precursor cells purified to greater than 99.95% purity), or mature oligodendrocytes (immature oligodendrocytes aged for 10 days), in the presence of insulin, forskolin and other factors as indicated and assessing cell survival after 3 days of culture.

The results are summarized in Table 3. In the presence of BDNF and CNTF, the RGCs cultured in the presence of oligodendrocytes, particularly mature oligodendrocytes, had significantly higher rates of survival at three days (~79%) than RGCs cultured in the presence of growth factors and forskolin alone (~55%).

The above results suggest that whereas a combination of forskolin, BDNF, CNTF and IGF-1 promote the long-term survival of about 50 to 60% of the purified retinal ganglion cells, an additional signal made by oligodendrocytes increases the percent of surviving cells to approximately 80%.

The results further indicate that the activity of the oligodendrocyte-derived signal (referred to as oligodendrocyte-derived neurotrophic factor; ODNF) is not mimicked by other known

factors that have been tested (NGF, NT-3, TGF-β1, TGF-β2, TGF-β3, glial cell-derived neurotrophic factor (GDNF), IL-7, IL-3, growth hormone (GH) and glial growth factor (GGF)).

ODNF was further characterized as detailed in Example 9. Serum-free MBS medium containing was conditioned by a dense culture of mature oligodendrocytes for at least three days, and subjected to one of three treatments. One sample of the serum was boiled for 5 minutes to heat-inactivate proteinaceous activity, another sample was incubated in a solution of trypsin-coupled beads, and a third sample was centrifuged through a concentrator membrane which excluded molecules greater than 10 kD. These medium samples were then tested for their ability to promote survival purified P8 retinal ganglion cells plated on merosin-coated wells in a 96-well plate as described in Example 9.

The results are shown in Table 5. Trypsin digestion and boiling both inactivated the survival-promoting activity (ODNF) in the medium. The survival activity was present in the $>$ 10kDa fraction retained on the filter, but not in the filtrate (containing molecules $<$ 10 kDa). The results suggest that oligodendrocyte-derived neurotrophic factor (ODNF) is a trypsin-sensitive heat-sensitive $>$ 10 kD proteinaceous factor secreted by oligodendrocytes.

One of skill in the art having the benefit of the present disclosure may proceed to obtaining the cDNA sequence encoding the factor using techniques known to those skilled in the art (*e.g.*, Ausubel, *et al.*,; Sambrook, *et al.*). For instance, Example 10 describes the synthesis of a cDNA library which may be used to isolate the ODNF cDNA using expression cloning. The library was prepared in the expression vector pMET7 (Takabe, 1988), containing the highly efficient SR alpha promoter (Kriegler, 1990), from poly-A^+ mRNA isolated from highly purified ($>$ 99.95% pure) mature oligodendrocytes. It contains over one million independent recombinants with an average insert size 1.6 kB. Other suitable expression vectors, such as pSG5 (Green, *et al.*, 1988; Stratagene, La Jolla, CA), may also be used for the construction of such a library.

A cDNA made from mature oligodendrocytes maybe screened using methods known to those skilled in the art to identify clones encoding ODNF. For example, an expression screening approach, such as is detailed in Example 10, may be employed. The library is amplified and divided into pools, from which groups of plasmids are isolated for transfection using lipofectamine (or DEAE Dextran) into eukaryotic cells, such as COS7 cells.

Following transfection, conditioned medium from the transfected cells is collected and assayed for survival-promoting activity (as described above) on purified postnatal RGCs. Pools of cells that enhance survival are then subcloned and rescreened until individual clones having survival activity are identified.

ODNF clones identified, for example, by the methods described above, maybe further characterized, manipulated and expressed to generate recombinant ODNF. For example, as

described in Example 11, cDNA clones encoding the RGC survival activity (ODNF) are isolated, sequenced, and the sequences compared for homologies to known sequences. Northern blot and *in situ* hybridization analyses may be performed to verify that the mRNAs are expressed specifically by oligodendrocytes but not astrocytes.

Recombinant ODNF polypeptides may be produced using a clone encoding the full-length ODNF (the clone may isolated as a full length clone or assembled from overlapping partial clones). The recombinant polypeptides may be prepared as fusion proteins, by cloning ODNF cDNA polynucleotide sequences into an expression plasmid, such as pGEX, to produce corresponding polypeptides. The plasmid pGEX (Smith, *et al.*, 1988) and its derivatives express the polypeptide sequences of a cloned insert fused in-frame with glutathione-S-transferase. The recombinant pGEX plasmids is transformed into an appropriate strains of *E. coli* and fusion protein production is induced by the addition of IPTG (isopropyl-thio galactopyranoside). Solubilized recombinant fusion protein is then purified from cell lysates of the induced cultures using glutathione agarose affinity chromatography according to standard methods (Ausubel, *et al.*).

Affinity chromatography may also be employed for isolating β-galactosidase fusion proteins. The fused protein is isolated by passing cell lysis material over a solid support having surface-bound anti-β-galactosidase antibody.

Isolated recombinant polypeptides produced as described above may be purified by standard protein purification procedures, such as differential precipitation, molecular sieve chromatography, ion-exchange chromatography, isoelectric focusing, gel electrophoresis and affinity chromatography, and used for subsequent experiments, such as generation of antibodies (Harlow, *et al.*). Antibodies directed against the RGC survival factor may be used for immunolocalization of this protein and/or to block the oligodendrocyte-derived survival activity.

The recombinant protein may also be used to promote the survival of retinal ganglion cells in culture, as well as to promote the survival and axonal regeneration of glutamatergic projection neurons, such as retinal ganglion cells, *in vivo* following neuronal injury.

The results discussed above indicate that oligodendrocytes synthesize and secrete a novel neurotrophic factor, and that this factor promotes the long-term survival of retinal ganglion cells. Since most other types of CNS neurons are myelinated by oligodendrocytes, it is contemplated that the oligodendrocyte neurotrophic factor promotes the survival of all such oligodendrocyte-myelinated CNS neurons. Accordingly, the oligodendrocyte-derived neurotrophic factor may be useful for the treatment of brain injuries, glaucoma, demyelinating diseases, and various neurodegenerative diseases that affect oligodendrocyte-myelinated CNS neurons.

For example, ODNF may be used in therapies involving repair and regeneration of neurons after brain injury (*e.g.*, evaluated as outlined in Example 12). Unlike many other tissues,

the central nervous system has little ability to repair itself. When the optic nerve or other myelinated tracts are injured in the CNS, the axons comprising these tracts are typically cut or damaged. The neurons whose axons are damaged fail to regenerate the axons and die, causing permanent neurological disability. In the case of the optic nerve, such neuronal cell death results in blindness. Damage to axon tracts after spinal cord injury results in paralysis.

Experiments performed in support of the present invention (Barres, *et al.*, 1993) demonstrate that when optic nerve axons are transected, optic nerve oligodendrocytes die. The results of experiments detailed herein suggest that just as retinal ganglion cells depend on optic nerve oligodendrocytes to survive, the oligodendrocytes require survival signals from retinal ganglion cells. Accordingly, it is contemplated that axotomized neurons die because they fail to get ODNF, and that delivery of exogenous ODNF to an injured brain region may be used to increase the percentage of injured neurons that survive and regenerate, thus alleviating some of the permanent neurological consequences (*e.g.*, paralysis, blindness) that were heretofore inevitably associated with CNS injury.

ODNF may also be employed in the treatment of glaucoma, a disease associated with increased intraocular pressure. One of the characteristics of glaucoma is the death of retinal ganglion cells. The reasons for such death are not fully understood. While not wishing to be bound by any particular molecular mechanism, the present invention contemplates that the retinal ganglion cells die because the elevated intraocular pressure blocks the retrograde transport of ODNF from the axons (where it is absorbed from oligodendrocytes) to the cell bodies, and accordingly, that intraocular application of ODNF may prevent retinal ganglion cell death caused by glaucoma.

Multiple sclerosis and other demyelinating diseases may also be treated using methods of the present invention. In the course of multiple sclerosis, oligodendrocytes die for unknown reason. Initially, new oligodendrocytes are generated, but as the disease progresses and more severe oligodendrocyte loss ensues, there is correspondingly less repair. It is known that sites of demyelination in multiple sclerosis are associated with axonal degeneration, suggesting that the neurons that used to project through the demyelinated region have died. The loss of such axons causes permanent paralysis because prior to the teachings herein, it has not been possible to stimulate the CNS to regenerate. The results of experiments presented in the present disclosure indicate that oligodendrocytes produce a potent neurotrophic factor may explain this axonal loss, since loss of oligodendrocytes would cause loss of their factor and ultimately death of the neurons that depend on this factor. Accordingly, it is contemplated that a primary problem in multiple sclerosis is not loss of oligodendrocytes, but loss of axons, and that delivery of exogenous ODNF into demyelinated brain regions may be employed to prevent such axonal loss, by preventing the death of the neurons that depended on oligodendrocytes for their survival.

Neurodegenerative diseases, such as Alzheimer's disease and amyotrophic lateral sclerosis (ALS) may also be treated using ODNF. In neurodegenerative diseases, neurons typically die for unknown reason. It is contemplated that delivery of exogenous oligodendrocyte trophic factor into the affected regions of brains of patients with neurodegenerative disease may be employed to treat or delay the course of their disease.

In addition to the therapeutic uses of synthetic or recombinant ODNF described above, the structure of the factor and/or information about biologically-active regions of the factor may be used to design peptide mimetics with increased stability, bioavailability and/or bioactivity relative to the native factor.

The following examples illustrate but in no way are intended to limit the present invention.

MATERIALS AND METHODS

Unless otherwise indicated, chemicals were purchased from Sigma (St. Louis, MO).

A. Buffers

Phosphate-buffered saline (PBS)

10x stock solution, 1 liter:

80 g NaCl

2 g KCl

11.5 g $Na_2HPO4\text{-}7H_2O$

2 g KH_2PO_4

Working solution, pH 7.3:

137 mM NaCl

2.7 mM KCl

4.3 mM $Na_2HPO_4\text{-}7H_2O$

1.4 mM KH_2PO_4

B. Animal Procedures

Cells for *in vitro* experiments were obtained by sacrificing the animals (typically rats) and obtaining the appropriate tissue by dissection. The animals were sacrificed either by fluothane inhalation anesthesia (adult rats) or by fluothane inhalation anesthesia followed by decapitation with a sharp pair of scissors (rat pups). For certain *in vivo* experiments (indicated), a minor surgical procedure (performed under fluothane inhalation anesthesia) involving optic nerve transection or intraocular injection was required.

C. Growth Factors

Recombinant human insulin-like growth factor 1 (IGF-1), basic fibroblast growth factor (bFGF), transforming growth factor-alpha (TGF-α) and transforming growth factor-beta (TGF-β) were obtained from Peprotech (Rocky Hill, NJ). Insulin was obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant mouse neurotrophin-3 (NT-3) was obtained from Yves Barde (Gotz, *et al.*, 1992, Max Plank Institute for Psychiatry, Martinsried, Federal Republic of Germany), recombinant human brain-derived neurotrophic factor (BDNF) and neurotrophin-4/5 (NT-4/5) from Regeneron Pharmaceuticals, Inc. (Tarrytown, NJ), recombinant rat ciliary neurotrophic factor (CNTF) from Michael Sendtner and Hans Thoenen (Stockli, *et al.*, 1989, Max Plank Institute for Psychiatry, Martinsried, Federal Republic of Germany), and recombinant human leukemia inhibitory factor (LIF) from John Heath (Heath and Smith, 1988, Oxford University, Oxford, England).

The growth factors may also be obtained from other commercial sources. For example, NT-3 may be obtained from Regeneron Pharmaceuticals; CNTF, LIF, IGF-1, IGF-2, bFGF and IL-6 may be obtained from Genzyme Diagnostics, Cambridge, MA; and LIF, TGF-α, IGF-1, IGF-2, bFGF and IL-6 may be obtained from R&D Systems (Minneapolis, MN). Forskolin and other activators of adenylate cyclase/protein kinase can be obtained from Calbiochem, La Jolla, CA; 3-isobutyl-1-methylxanthine and other phosphodiesterase inhibitors can be obtained from Calbiochem or other commercial sources.

D. Dissection of Retinas, Optic Nerve, Tectum and Neurocortex.

Retinal, optic nerve, tectal and cortical tissues were obtained from Sprague Dawley (S/D) rats (Simonsen Labs, Simonsen, CA). Retinas were removed from the eye *in situ* as follows. The animal was decapitated and skin overlying the eyes removed. The head was pinned to a block, and the anterior tissues of the eye were removed with a #11 scalpel blade while the cornea was held with a pair of rat-tooth, micro-dissecting forceps. The lens and vitreous humor were removed with forceps. The retina was then gently lifted away with a small spatula. Retinas were stored at room temperature in Eagle's Modified Essential Medium (MEM; Gibco/BRL Life Technologies, Gaithersburg, MD) until retinas were dissected from all animals.

Optic nerve and optic chiasm were dissected from postnatal day 8 (P8) S/D rats with micro-dissecting forceps and small scissors, collected in 35 mm petri dishes containing 2 ml of MEM supplemented with 10 mM Hepes (MEM/Hepes), and minced using small scissors.

The tectum was dissected with the aid of micro-dissecting forceps and a scalpel and temporarily stored in Hepes/MEM as above.

The neurocortex was dissected with the aid of micro-dissecting forceps and a scalpel and temporarily stored in Hepes/MEM as above.

E. Dissociation of Retina, Optic Nerve, Tectum and Neurocortex.

The retinal, optic nerve, tectal and cortical tissues dissected as above were dissociated enzymatically to make a suspension of single cells, essentially as described by Huettner and Baughman (1986).

A papain solution was prepared, immediately prior to the start of the dissection, by adding 150 units (retina, tectum and cortex) or 300 units (optic nerve) of papain (Worthington Biochemical, Freehold, NJ) to 10 ml of Earle's Balanced Salt Solution (EBSS; Gibco/BRL Life Technologies, Gaithersburg, MD) in a 15 ml blue-top conical centrifuge tube, and placing the mixture in a 37°C water bath to dissolve the papain. One hundred microliters of a 4 mg/ml DNAse (0.004%, Worthington Biochemical Corp., Freehold, NJ) solution were added to the MEM/papain mixture after the papain had dissolved. About 10 minutes before use, the solution was mixed with 2.4 mg of L-cysteine, adjusted to a pH of about 7.4 with 1M NaOH, and passed through a 0.22 micron filter into sterilized scintillation vials.

Upon completion of the dissection (described in section D, above), the MEM bathing the tissue was removed with a sterile pasteur pipette and replaced with 2 ml of the papain solution. The tissue was then decanted to a scintillation vial containing fresh papain solution, and the vial was placed in a 37°C water bath for 30 minutes with gently swirling approximately every 10 minutes.

The tissue and papain solution in the scintillation vial were then decanted to a 15 ml blue-top centrifuge tube. After the tissue settled to the bottom, the old papain solution was removed with a sterile pipet and the tissue was gently rinsed with 3 ml of ovomucoid inhibitor solution, which contained ovomucoid (15 mg; Boehringer-Mannheim) and BSA (10 mg; Sigma catalog no. A-7638) dissolved in MEM (GIBCO/BRL). The solution was adjusted to pH 7.4 and sterilized with a 0.22 μm filter). The pieces of tissue were allowed to settle, and the rinse solution removed.

Retinal, tectal and cortical tissue was then triturated sequentially with a 1 ml plastic pipette tip (Rainin Instr. Co., Woburn, MA), while optic nerve tissue was triturated with #21 and then #23 gauge needles. The following steps were repeated 6-10 times (until the tissue was completely broken up): (i) one ml of ovomucoid solution was added and the tissue was gently pulled up into the pipet and expelled, (ii) the dissociate was allowed to settle by gravity for about 30 seconds, and (iii) the supernatant was collected.

The final cell suspension, comprised of the supernatants from the 6-10 trituration cycles, contained about 20 million cells per P8 retina, about 50,000 cells per P8 optic nerve, about 10^6 cells per P0 tectum, and about 10^7 cells per P8 cortex.

The cell suspension was then spun at 800 X*g* for 10 minutes in a 15 ml blue-top centrifuge tube to separate the cells from the ovomucoid solution. The supernatant was discarded

and cells resuspended in 1 ml of MEM. The cell suspension was then gently layered onto 6 ml of an MEM solution containing 60 mg of ovomucoid and 60 mg of BSA (pH adjusted to pH 7.4) and spun again at 800 X*g* for 10 minutes in a 15 ml blue-top centrifuge tube. The supernatant was discarded and the cells were resuspended in 12 ml of Eagle's Minimum Essential Medium (MEM) solution containing BSA (0.1%).

During this procedure the cells were never exposed to glutamate, aspartate or glutamine, or allowed to be cooled lower than room temperature.

F. Purification of Retinal Ganglion Cells

Retinal ganglion cells were purified from the retinas and optic nerves of E18 or P8 rats using a panning procedure (Barres, *et al.*, 1988; Barres and Chun, 1993). A brief description of the procedure follows.

1. Preparation of Panning Plates. Two sets of panning plates, either 100 mm × 15 mm or 150 mm × 15 mm petri dishes (Fisher Scientific, Pittsburgh, PA; Cat No. 8-757-12), were prepared as described below. Dishes made of tissue culture plastic were not used due to potential problems with non-specific cell sticking. None of the incubation solutions used to coat panning plates were sterilized with 0.22 μm filters, since much of the protein would have been lost in the filter.

Panning plates comprising the first set were incubated with 5 ml of 50 mM Tris buffer (pH 9.5) containing 0.5 μg/ml affinity-purified goat anti-rabbit IgG (H+L; Accurate Chemical & Scientific Corp., Westbury, NY) for 12 hours at 4°C. The supernatant was removed and the dishes were washed three times with 8 ml of PBS. To prevent nonspecific binding of cells to the panning dish, 5 to 15 ml of Eagle's MEM with BSA (0.2%) was placed on each dish for at least 20 minutes, and this solution remained on the dish until it was used.

Petri dishes in the second set were prepared as above, except that the Tris buffer contained 5 μg/ml affinity-purified goat anti-mouse IgM (mu-chain specific; Accurate Chemical & Scientific Corp., Westbury, NY). The dishes were then washed as above and incubated with 5 ml of a supernatant from mouse monoclonal cell line T11D7e2, containing IgM antibodies against Thy1.1, for at least one hour at room temperature. The supernatant was removed and the plate washed three times with PBS. In order to prevent nonspecific binding of cells to the panning dish, 5 ml of Minimal Essential Medium (MEM; Gibco/BRL) with 2 mg/ml BSA was placed on the plate for at least 20 minutes.

Cell line T11D7e2 is available from the American Type Culture Collection (ATCC; Rockville, MD; Accession number TIB 103).

2. Panning Procedure. The panning procedure is summarized schematically in Figures 1A-1E. A retinal cell suspension (**20**) prepared as above and containing retinal ganglion cells (**22**), macrophages (**24**) and various other cells, including Thy1 negative cells (**26**), was incubated with antiserum containing rabbit-anti-rat-macrophage antibodies (**28**; Accurate Chemical & Scientific Corp., 1:100) for 20 minutes (Fig. 1A), centrifuged at 800 *Xg* for 10 minutes, resuspended in MEM, and incubated on a goat-anti-rabbit IgG (**30**) (first set) panning plate (**32**; 150 mm) at room temperature for 45 minutes (Fig. 1B). The plate was gently swirled after 20 minutes to ensure access of all cells to the surface of the plate. If cells from more than 8 retinas were panned, the nonadherent cells were transferred to another (first set) 150-mm anti-rabbit IgG panning plate for an additional 30 minutes.

Non-adherent cells were removed with the suspension, filtered through a UV-sterilized 15 micron Nitex mesh (Tetko, Elmsford, NY) to remove small clumps of cells, placed on a second set panning plate (**34**) derivatized with goat-anti-mouse IgM (**36**) and mouse anti-Thy-1 (**38**; T11D7), and incubated on the plate (Fig. 1C) as described above. After 1 hour, the plate was washed eight times with 6 ml of PBS and swirled moderately vigorously to dislodge nonadherent cells (Fig. 1D).

When solutions were removed from the panning dishes during washes, they were immediately replaced with fresh solution so that the cells did not dry out. Progress of nonadherent cell removal was monitored under a microscope. The washing was terminated when only adherent cells remained.

3. Removing Adherent Cells from the Plate. Four ml of a trypsin solution (0.125%) were prepared by diluting a trypsin 20X stock (Sigma) into EBSS. Cells adhering to the panning dish (**34**) were incubated with this solution for 10 minutes in a 5% CO_2 incubator at 37°C. The cells were dislodged by gently pipetting trypsin solution around the plate. Ten ml of a 25% fetal calf serum (FCS; Gibco/BRL) solution were added to inactivate the trypsin and the cells (Fig. 1E) were spun and collected as above. To eliminate traces of FCS, the cells were resuspended and spun down again in an MEM solution containing BSA (0.5%). The cells were then resuspended in MBS culture medium for use in the experiments.

G. Purification of O-2A Progenitor Cells, Oligodendrocytes and Astrocytes.

Oligodendrocytes and their precursors were purified from the optic nerves of P8 S/D rats as previously described (Barres, *et al.*, 1992; Barres, 1993).

1. Preparation of Panning Plates. Three sets of panning plates were prepared. The first set was incubated with affinity-purified goat anti-mouse IgG (H+L chain

specific, Accurate Chemical & Scientific Corp., Westbury, NY) as above, washed as above, and further incubated with anti-RAN-2 IgG monoclonal antibody (supernatant 1:4; Bartlett, *et al.*, 1981) as above. RAN-2 is an unknown protein that is specifically expressed on optic nerve type-1 astrocytes (Bartlett, *et al.*, 1981).

The second and third sets of panning plates were both incubated with affinity-purified goat anti-mouse IgM (mu-chain specific, Accurate), as above, washed, and further incubated with either anti-GC (anti-galactocerebroside glycolipid) monoclonal IgG antibody (RMab supernatant 1:4; Ranscht, *et al.*, 1982; second set) or with anti-A2B5 monoclonal IgM ascites at 1:2000 (Eisenbarth, *et al.*, 1979; third set). A2B5 is a ganglioside specifically expressed on oligodendrocyte precursor cells, while galactocerebroside glycolipid is specifically expressed on oligodendrocytes. The antibodies were diluted in Hepes-buffered Minimal Eagle's Medium (MEM/Hepes, Gibco/BRL) containing bovine serum albumin (BSA, 1mg/ml; Sigma A4161), in order to block the non-specific adherence of cells to the panning plates. The antibody solution was removed, the plates washed three times with PBS, and PBS left on the plates until use.

2. Immunopanning Procedure. The optic nerve cell suspension was resuspended in 7 ml of L15 Air Medium (Gibco/BRL), containing insulin (5 μg/ml; Sigma Chemical) and filtered through UV-sterilized Nitex mesh (15 μm pore size, Tetko). The cell suspension was placed on a RAN-2 (first set) plate for 30 minutes at room temperature, with brief and gentle agitation after 15 minutes, to deplete type-1 astrocytes, meningeal cells, microglia and macrophages. The non-adherent cells were transferred to a second RAN-2 plate for 30 minutes, after which the non-adherent cells were transferred to a (second set) GC plate for 45 minutes to deplete oligodendrocytes.

Cells that did not adhere to the GC plate were transferred to a (third set) A2B5 plate and incubated as above to deplete the O-2A progenitor cells. Non-adherent cells were discarded.

The second and third set plates, containing adherent GC^+ oligodendrocytes and $A2B5^+$ O-2A progenitors, respectively, were washed 8 times with 6 ml of PBS or MEM/Hepes with moderately vigorous agitation to remove all antigen-negative non-adherent cells. The progress of nonadherent cell removal was monitored under an inverted phase-contrast microscope, and washing was terminated when only adherent cells remained.

The purification procedure for optic nerve type-1 astrocytes followed a similar protocol. Optic nerves were dissected from P2 rats. The first panning dish was coated with the MRC-OX7 Thy1.1 antibody (IgG; Serotec Ltd., Station Approach, UK) to deplete the suspension of meningeal fibroblasts, the second dish was coated with A2B5 and GC antibodies (as above) to deplete the suspension of O-2A lineage cells, and the final dish was coated with the C5 monoclonal antibody (Miller, *et al.*, 1984) directed against an unknown antigen on neuroepithelial

cells to select any remaining neuroepithelial cells, which were greater than 99.5% GFAP-positive type-1 astrocytes. The astrocytes were released from the C5 plate using a trypsin solution as described above.

H. Purification of Glutamatergic Cortical Projection Neurons by Immunopanning

Glutamatergic cortical projection neurons were purified from the neurocortex of P8 rats following the protocol for retinal ganglion cell purification described above.

I. Culture of Purified Retinal Ganglion Cells

Approximately 5,000 purified retinal ganglion cells (RGCs) were cultured in 96-well plates (Falcon) that had been coated with merosin (2 μg/ml; Telios Pharmaceuticals Inc., San Diego, CA, available from Gibco/BRL) in 100 μl of modified Bottenstein-Sato (MBS) serum-free medium. The percentage of surviving cells was assessed after 3, 7, and 14 days by the MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (see below). All values were normalized to the percentage of surviving cells at 3 hours after plating, which represented the percentage of cells that survived the purification procedure. This initial viability was typically about 85%.

The MBS medium was similar to Bottenstein-Sato (B-S) medium (Bottenstein and Sato, 1979), but used "NEUROBASAL" (Gibco/BRL), instead of Dulbecco's Modified Eagle's Medium (DMEM), as the base. "NEUROBASAL" is a recently-described basal medium that has been optimized for neuronal cell culture (Brewer, *et al.*, 1993). Media employing "NEUROBASAL" promoted better survival of RGCs than those employing DMEM, and differed from those using DMEM primarily in that they had a lower osmolarity (235 mOsm for "NEUROBASAL" vs. 335 mOsm for DMEM). The final osmolarity of the MBS "NEUROBASAL"-based culture medium used in the present experiments, after addition of the serum-free additives, was about 260 mOsm.

The serum-free components added to the "NEUROBASAL" base to make MBS medium were bovine serum albumin (BSA), selenium, putrescine, thyroxine, tri-iodothyronine, transferrin, progesterone, pyruvate and glutamine. Various trophic factors and other additives were added as indicated in individual experiments. The MBS medium was prepared with a highly purified, crystalline grade of BSA (Sigma, A4161), in order to avoid contaminating survival factors.

The component concentrations of the MBS medium used in the present experiments are provided in Table 1, below.

Table 1

MBS COMPONENTS

Component	Amount/Conc.
bovine serum albumin (BSA)	100 μg/ml
sodium selenite	40 ng/ml
putrescine	16 μg/ml
thyroxine	40 ng/ml
tri-iodothyronine	30 ng/ml
transferrin	100 μg/ml
progesterone	60 ng/ml
pyruvate	1 mM
glutamine	1 mM

J. Preparation of Co-cultures and Conditioned Medium

Co-culture experiments were performed to assess the effects of soluble factors released by retinal cells, tectal cells, optic nerve cells, and purified astrocytes or oligodendrocytes on RGC survival. Approximately 50,000 cells were plated onto the bottom of poly-d-lysine or merosin-coated wells in a 24-well plate. The cells were incubated at 37°C under a 10% CO_2 atmosphere for at least three days to condition serum-free Sato medium, which, depending on the experiment, may have contained additional trophic factors (indicated). In some cases (indicated), purified oligodendrocytes were allowed to mature for 10 days prior to further use.

Three small sterile glass chips were placed at the bottom of each well to act as pedestals to support a 13 mm merosin-coated glass coverslip. After three days, the glass coverslip was added to each well and 10,000 purified retinal ganglion cells were plated onto each slip. Each well thus contained two layers of cells that did not contact one another, with the conditioning cells comprising the bottom layer and the purified RGCs comprising the top layer. The medium was changed by replacing half of the volume of the well with fresh medium every three days.

K. MTT Survival Assay

The MTT survival assay was performed as described by Mosmann (1983). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma) was dissolved in PBS at 5 mg/ml and sterilized by passage through a 0.22 μm Millipore filter (VWR Scientific Corp., Westchester,

PA). This stock solution was added to the culture well (1:9) and incubated at 37°C for 1 hour. Viable cells with active mitochondria cleaved the tetrazolium ring of MTT into a visible dark blue formazan reaction product. The viable and dead cells in each well were counted by bright-field microscopy.

L. Immunofluorescence Staining

Cells were fixed with 4% paraformaldehyde for 5 minutes at room temperature. Non-specific binding was blocked by a 30 minute incubation in 50% goat serum containing 1% BSA and 100 mM l-lysine. The cells were then surface-stained, either with monoclonal anti-GC antibody (supernatant used at 1:1) followed by fluorescein-coupled goat anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA; 10 μg/ml) or with A2B5 antibody (supernatant diluted 1:1) followed by fluorescein-coupled goat anti-mouse IgM (μ chain specific, Jackson; 10 μg/ml).

In order to stain intracellular antigens or compounds, cells were permeabilized by adding "TRITON" X-100 (0.4%) to the goat serum solution. Cells were stained with rabbit anti-GFAP antiserum (DAKO Corp., Carpinteria, CA; diluted 1:100). Anti-GFAP antibodies were detected with fluorescein-coupled goat anti-rabbit IgG (H+L chain specific, Accurate Chemical & Scientific Corp.). In order to stain the cytoskeletal proteins in dendrites and axons, cells were incubated in monoclonal anti-MAP-2 antibody (Boehringer-Mannheim; 5 μg/ml) or in monoclonal anti-tau antibody (Boehringer-Mannheim; 5 μg/ml), respectively, followed by fluorescein-coupled goat anti-mouse IgG (Jackson, 10 μg/ml). The coverslips were mounted in "CITIFLOUR" (University of London, Canterbury, England) on glass slides, sealed with nail varnish and examined in a Zeiss Axioskope fluorescence microscope (Carl Zeiss Inc., Thornwood, NY). Glial cell types were identified by their characteristic antigenic phenotypes: astrocytes were labeled by anti-GFAP antiserum, oligodendrocyte precursor cells by A2B5 antibody and oligodendrocytes by anti-GC antibody (Ranscht, *et al.*, 1982). Anti-cAMP antibodies (T. Wielmelt and A. McMorris, also available from Sigma Immunochemicals, St. Louis, MO) were used to detect elevation of adenosine 3'5'-cyclic monophosphate (cAMP). Translocation of MAP kinase to the nucleus was carried out by first permeabilizing membranes with Triton X100, as described above, followed by exposure to anti-MAP kinase (ERK) antibody (Calbiochem, La Jolla, CA) for one hour, and subsequent exposure to fluorescein-conjugated anti-rabbit antibody, according to methods known in the art.

M. Electrophysiological Recording

Standard procedures for preparing pipets, forming seals, and whole-cell patch recording using an Axopatch 200A patch clamp (Axon Instruments, Burlingame, CA) were utilized (Hamill,

et al., 1981). Micropipets were drawn from hard borosilicate capillary glass (VWR Scientific Corp.) and fire-polished to an internal tip diameter of about 1 micron. Pipet capacitance and series resistance were electronically compensated with the patch clamp. All experiments were performed at room temperature (approximately 24°C). The bath solution contained (in mM) NaCl 140, $CaCl_2$ 2, $MgCl_2$ 1, KCl 5, Hepes 5, pH 7.4. The pipet solution contained (in mM) KCl 140, $MgCl_2$ 1, Ca^{++} buffered to 10^{-6} M with EGTA 10, Hepes 5, pH 7.4.

EXAMPLE 1

Effects of Growth Factors on Survival of Purified Postnatal Retinal Ganglion Cells in Culture

Approximately 5,000 purified P8 retinal ganglion cells were plated in triplicate in merosin-coated 96-well plates (Falcon) in 100 μl of MBS medium without insulin, containing a plateau concentration of the indicated factor (50 ng/ml for all factors except for insulin, which was 5 μg/ml). After 3 days (3D) of culture, the percentage of cells surviving in each well was determined using the MTT assay (means $\pm$ S.E.M.; see Materials and Methods).

A. Effects of Single Factors and cAMP Elevation

To determine the effects of individual growth factors on the survival of retinal ganglion cells, the factors were applied in isolation (one factor per experiment) to the P8 cultures described above, either with or without 5 μM forskolin. The results are summarized in Table 2, below.

Table 2

SURVIVAL OF PURIFIED RETINAL GANGLION CELLS IN CULTURE

Factor	% Surviving Cells at 3D No Forskolin	% Surviving Cells at 3D Forskolin
Nothing	0.8 ± 0.3	5.5 ± 1.0
Insulin	1.3 ± 0.4	16.2 ± 1.5
IGF-1	1.5 ± 0.5	18.3 ± 1.2
BDNF	2.4 ± 0.5	35.8 ± 2.6
NT-4/5	2.1 + 0.4	32.9 + 2.8
CNTF	1.9 ± 0.6	29.7 ± 2.5
LIF	1.5 ± 0.4	28.1 ± 1.7
bFGF	2.0 ± 0.4	17.2 ± 1.9
TGF-α	1.2 ± 0.5	13.3 ± 2.1

As can be appreciated from the above results, high (plateau) concentrations of single peptide growth factors, including insulin, insulin-like growth factor 1 (IGF-1), brain-derived

neurotrophic factor (BDNF), neurotrophin-4/5 (NT-4/5), ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), basic fibroblast growth factor (bFGF), and transforming growth factor-alpha (TGF-α), did not promote the survival of more than about 2 % of cells. Similar results were obtained with interleukin-6 (IL-6).

However, elevating intracellular cAMP levels (either by addition of forskolin, an activator of adenylate cyclase, or by the addition of the membrane permeable cAMP analog, chlorophenylthio-cAMP (CPTcAMP)), amplified by about 10-fold the ability of individual peptide trophic factors to promote RGC survival. Neither forskolin nor CPTcAMP had significant survival promoting effects when applied alone (in the absence of trophic factors). Factors effective at promoting survival under conditions of elevated cAMP included IGF-1, IGF-2 and insulin at concentrations sufficiently high to activate IGF-1 receptors (5 μg/ml; Sara and Hall, 1990), as well as BDNF, NT-4/5, CNTF, LIF, IL-6, bFGF, and TGF-α.

The above results indicate that the short-term survival (survival at 3 days of culture) of RGCs can be promoted by the application of single trophic factors in combination with cAMP-elevating agents or cAMP analogs (acting, as described below, to stimulate cAMP-dependent protein kinase). The addition of factors effective to stimulate cAMP-dependent protein kinase elevated the survival at 3 days from <3% to between 15 and 40%.

Trophic factors which at high concentration (50 ng/ml) did not promote the survival of RGCs in the presence of forskolin included NT-3, nerve growth factor (NGF), transforming growth factors beta 1, 2, and 3, glial cell-derived neurotrophic factor (GDNF), glial growth factor (GGF), interleukin-3 (IL-3) and interleukin-7 (IL-7). While such factors may not promote survival of retinal cells at low concentrations, it is appreciated that they may be useful at higher concentrations and/or for different neuronal cell types. Experimentation to determine whether these and other related factors are useful for a particular cell type is within the skill of the practitioner provided the teachings herein, particularly using as a model, the exemplary teachings with regard to testing retinal cells, above.

The survival promoting effects of different concentrations of BDNF and CNTF on purified RGCs were determined using dose-response curves in medium containing forskolin (5 μM) and insulin (5 μg/ml). Figure 2A shows dose-response curves for BDNF and CNTF. The BDNF concentration that promoted half-maximal survival was about 2 ng/ml and for CNTF was about 200 pg/ml, similar to the values reported for their survival promoting effects on rodent peripheral neurons (Hughes, *et al.*, 1993; Arakawa, *et al.*, 1990).

B. Effects of Multiple Factors

Experiments were performed to determine whether combinations of the factors tested improve short-term survival when applied simultaneously. The factors were grouped into three

classes: (i) insulin-like growth factors (*e.g.*, insulin, IGF-1, IGF-2), (ii) neurotrophins (*e.g.*, BDNF and NT-4/5), and (iii) CNTF-like cytokines (*e.g.*, CNTF, LIF, and IL-6). Combinations of two or three factors from within a class did not give a significantly better survival than the individual factors alone. In contrast, a combination of factors from different classes produced additive effects on survival.

Exemplary results are summarized in Figure 2B, which shows the effects of combining survival factors on short term (3 day) retinal ganglion cell survival. All factors were used at plateau concentrations. Insulin plus BDNF, for example, was better than insulin or BDNF alone, while insulin plus BDNF and CNTF was better than any two of these alone. The majority of the cells could be saved, at least for 3 days, by the combination of forskolin, insulin, BDNF, CNTF and growth on a merosin-coated surface. The addition of bFGF and TGF-α did not promote a further increase in survival, but additive effects were observed when these factors were combined with CNTF. The omission of merosin (or laminin, whose effects were indistinguishable from those of merosin) as the substrate, even if replaced with poly-d-lysine, resulted in significantly lower % survival.

Combinations of peptide factors applied in the absence of forskolin promoted a much lower degree of survival than was obtained in the presence of forskolin. For example, survival in BDNF and insulin was about 10% but increased to 55% when forskolin was present; survival in BDNF, CNTF, and insulin was about 30% but increased to 80% with forskolin.

C. Long Term Survival

The experiments described in Example 1B, above, were carried out over 28 days to determine whether long-term survival of RGCs could be promoted by combinations of peptide trophic factors and cAMP elevating agents. The survival of purified retinal ganglion cells cultured in the indicated factors was assessed after 3, 7, 14 and 28 days of culture. The medium also contained forskolin (5 μM) and insulin (5 μg/ml).

Exemplary results are shown in Figure 3. By two weeks in culture, the majority of cells had died when cultured in only one or two factors, for instance BDNF and insulin. When all three factors were combined together (BDNF, CNTF, and insulin), the cells that survived for one week (about 63% of cells) were able to survive for at least 1 month. In contrast, after 1 month of culture, the majority of cells cultured in either BDNF and insulin or in CNTF and insulin had died, strongly suggesting that BDNF and CNTF do not promote the survival of different subsets of cells, but act synergistically on individual cells to promote long-term survival.

EXAMPLE 2

Effects of Cell Density on Postnatal Retinal Ganglion Cell Survival

The effects of cell density on survival were determined using purified retinal ganglion cells cultured in serum-free MBS medium containing forskolin and plateau concentrations of BDNF, CNTF, insulin, and in some cases bFGF, as indicated for 3 days or 7 days. The percentage of cells surviving was determined by the MTT assay.

Exemplary results are shown in Figs 4A (3 day culture) and 4B (7 day culture). In Fig. 4A, the cells were cultured either at high density (50,000 cells/well), low density (1500 cells per well) or at low density above a conditioning layer of high density cells (see Materials and Methods).

After 3 days in culture, survival at low density (1500 cells per well) was nearly three fold lower than at high density (50,000 cells per well). To determine whether this effect was soluble or contact-mediated, RGCs were cultured at low density on a glass coverslip positioned above a conditioning layer of high density RGCs as described in the Materials and Methods. The survival of the low density cells on glass coverslips with a conditioning layer (Low over High; Fig. 4A) was nearly identical to the survival of RGCs plated on glass coverslips at high density (Fig. 4A), suggesting that retinal ganglion cells secrete soluble signal(s) that promote their own survival.

To determine whether the effects of cell density on survival were due to basic fibroblast growth factor (bFGF), which is expressed by retinal ganglion cells *in vivo* (Elde, *et al.*, 1991; Connolly, *et al.*, 1992), the effects of bFGF on cells cultured at low density were examined. Cells were cultured at high and low density both in the presence and absence of bFGF. Exemplary results are summarized in Figure 4B. The survival of cells at low density was increased by the inclusion of bFGF, but not by TGF-α, in the culture medium, whereas the survival at high density was not significantly increased by the addition of bFGF. These results suggest that at least one of the survival factors secreted by RGCs to promote their own survival is bFGF. Further experiments indicated that the addition of bFGF to the medium increased the survival of RGCs at clonal density ($\sim$100 cells/60 mm dish) by 5-fold, with many cells surviving for weeks.

EXAMPLE 3

Effects of Glial Cells on Purified Postnatal Retinal Ganglion Cell Survival

The effects of visual pathway glia on survival of RGC cells was determined using conditioning layers of glial cells. Purified P8 RGCs were cultured over a conditioning layer of highly purified postnatal optic nerve astrocytes (purified to greater than 99.5% purity by

sequential immunopanning; see Materials and Methods). Cell survival was measured after 3 days using the MTT assay. The results are summarized in Table 3, below. In serum-free MBS medium containing only forskolin and insulin but no BDNF or CNTF, the astrocytes weakly promoted RGC survival. In the presence of BDNF and CNTF, however, there was little further additive effect.

To determine whether oligodendrocytes promote RGC survival, purified P8 RGCs were cultured as above over a conditioning layer of immature or mature oligodendrocytes. Immature oligodendrocytes were oligodendrocyte precursor cells that were purified to greater than 99.95% purity by sequential immunopanning from P8 optic nerve as described above and in Barres, *et al.*, 1992. Mature oligodendrocytes were prepared by aging the immature oligodendrocytes for 10 days.

Approximately 15,000 purified P8 retinal ganglion cells were plated in triplicate onto merosin-coated 12-mm glass coverslips mounted over a conditioning layer of glial cells (see Materials and Methods) in 24-well plates (Falcon) in 700 μl of MBS medium containing insulin (5 μg/ml) and forskolin (5 μM) with or without plateau concentrations of BDNF (50 ng/ml) and CNTF (50 ng/ml). After 3 days of culture, the percentage of retinal ganglion cells surviving in each well was determined using the MTT assay (means ± S.E.M.). As described above, it will be appreciated that for any given medium composition, the survival of RGCs cultured on glass coverslips is lower than their survival on plastic.

The results are summarized in Table 3, below. In serum-free MBS medium containing only forskolin and insulin, but no BDNF or CNTF, neither mature nor immature oligodendrocytes significantly promoted RGC survival. In the presence of BDNF and CNTF, however, there was a significant additive effect, particularly by mature oligodendrocytes. Under these conditions, the survival of the majority of retinal ganglion cells could be promoted for at least one month.

Table 3

EFFECTS OF GLIAL CELLS ON THE SURVIVAL OF PURIFIED RETINAL GANGLION CELLS IN CULTURE

Conditioning Cell Type	% Surviving Cells Forskolin Only (3D)	% Surviving Cells Forskolin+BDNF+CNTF +INS (3D)
None	15.3 ± 0.5	55.0 ± 2.2
Astrocytes	24.5 ± 0.8	54.0 ± 4.0
Immature Oligodendrocytes	17.9 ± 2.1	66.2 ± 4.2
Mature Oligodendrocytes	14.4 ± 0.5	79.0 ± 3.1

Morphological correlates of the above results are illustrated in Figures 5A and 5B, which show exemplary photomicrographs of purified P8 retinal ganglion cells cultured for 14 days in the absence (Fig. 5A) or presence (Fig. 5B) of a conditioning layer of mature oligodendrocytes. The cells were cultured in serum-free MBS medium containing plateau concentrations of BDNF, CNTF and IGF-1 on merosin-coated glass coverslips. Most of the retinal ganglion cells grown under the conditions shown in Fig. 5A died by two weeks in culture. In contrast, the majority of cells grown over the oligodendrocytes extended processes and survived for at least four weeks.

EXAMPLE 4

Effects of Growth Factors on Embryonic Retinal Ganglion Cell Survival in Culture

To determine whether the signals that promote the survival of postnatal RGCs also promote the survival of embryonic day 18 (E18) RGCs, most of which have not yet innervated the tectum, E18 RGCs were purified and cultured in MBS serum-free medium containing various peptide trophic factors as described above.

The survival of purified E18 retinal ganglion cells cultured in serum-free MBS medium containing high insulin (5 μg/ml) and various survival factors (50 ng/ml; used as indicated) assessed after 3 days of culture by the MTT assay is shown in Figure 6. BDNF as well as NT-4/5 significantly promoted the survival of the embryonic cells if forskolin was present in the medium. In contrast to the postnatal RGCs, however, there was little effect of CNTF or oligodendrocyte conditioned medium. Even in combination with forskolin and insulin, all of the other factors tested, including NGF, NT-3, bFGF, TGF-α, TGF-β1, TGF-β2, TGF-β3, LIF and GDNF, were without significant effect on purified E18 RGCs.

A comparison of the effects of various survival factors on embryonic and postnatal retinal ganglion cells is shown in Table 4, below.

Table 4

EFFECTS OF SURVIVAL FACTORS ON EMBRYONIC AND POSTNATAL RETINAL GANGLION CELLS

Factor	Embryonic RGCs	Postnatal RGCs
Require cAMP or depol.	Yes	Yes
BDNF (or NT-4/5)	Yes	Yes
E18 Tectal Factor	Yes	n.d.
CNTF	No	Yes
LIF	No	Yes
bFGF	No	Yes
TGF-α	No	Yes
RGC Paracrine Factor	No	Yes
Oligodendrocyte-derived Neurotrophic Factor	No	Yes

n.d. - not determined

Because it has been reported that the responsiveness to embryonic and postnatal RGCs to merosin differ (Cohen and Johnson, 1991), the effects of a laminin substrate were also tested. The results were similar to those obtained with merosin.

EXAMPLE 5

Effects of Tectal Conditioned Medium on Embryonic Retinal Ganglion Cell Survival in Culture

Although the embryonic RGCs responded to BDNF, most died after several days. To determine whether the neighboring cell types of embryonic RGCs make other soluble survival signals that act alone or in combination with BDNF, the effects of conditioned medium from E18 retina, optic nerve, and tectum on the survival of purified E18 RGCs in serum-free MBS medium containing BDNF, CNTF, insulin and forskolin were assessed. The CNTF, insulin and forskolin were added in part to promote survival of the conditioning cells.

The survival of the purified E18 retinal ganglion cells was assessed using the MTT assay after 3 days of culture above a conditioning layer of various cell types in serum-free MBS medium containing forskolin (5 μM) and plateau levels of BDNF, CNTF, and insulin. The conditioning

retinal, optic nerve and tectal cells were isolated from E18 rats, whereas the oligodendrocytes were isolated from P8 rats and matured for 10 days prior to the assay.

The results are summarized in Figure 7. The survival of the E18 RGCs was enhanced nearly 5-fold (relative to no treatment) by soluble tectal factors. The results indicate that the tectum secretes a significant survival activity which, according to additional experiments performed in support of the present invention, was not mimicked by known neurotrophins, including NGF, BDNF, NT-3, and NT4/5.

The morphology of purified E18 retinal ganglion cells cultured in serum-free MBS medium containing plateau concentrations of BDNF, CNTF, IGF-1 on merosin-coated wells in a 96-well plate is shown in Figs. 8A and 8B. The medium bathing the cells in Fig. 8A contained only the aforementioned components, while the medium bathing the cells in Fig. 8B also contained conditioned medium from E18 tectal cells (in a 1:1 ratio). Most of the cells grown under the conditions shown in Fig. 8A died by three days of culture. The majority of cells grown with tectal conditioned medium, however, extended processes and survived for at least 3 days (the longest time point assayed; Fig. 8B.

EXAMPLE 6

Effects of Depolarization on Postnatal Retinal Ganglion Cell Survival in Culture

The apparent responsiveness of both embryonic and postnatal RGCs to the effective peptide trophic factors tested was strongly dependent on elevation of their intracellular cAMP levels (accomplished in most of the aforementioned experiments by forskolin), suggesting the possibility that an additional substance, activated by elevated intracellular cAMP, acts collaboratively with peptide trophic factors to promote RGC survival. Continuous conditioning of medium by retinal, optic nerve and tectal cells, in the absence of cAMP elevating agents, failed to significantly promote the survival of purified RGCs. These results strongly suggest that these cell types do not secrete signals that increase RGC cAMP levels.

Electrical physiological recordings and calcium imaging using the calcium-sensitive dye fura-2 demonstrated that the purified P8 RGCs in culture have only low levels of spontaneous electrical activity, suggesting that electrical activity may be necessary for the RGCs to respond to their trophic factors. To test this possibility, potassium chloride was added to the culture medium in order to depolarize the RGCs.

Purified P8 retinal ganglion cells were cultured in serum-free MBS medium for 3 days in the indicated survival factors. In some cases, either forskolin (5 μM) or potassium chloride (10 or 50 mM as indicated; when not indicated, the results for 10 and 50 mM were not significantly different) was also included in the medium.

Results of these experiments are shown in Figure 9A. Although by itself depolarization did not promote RGC survival, it had an effect similar to that of increasing cAMP -- that is, depolarization markedly increased the efficacy of trophic factors (*e.g.*, BDNF) at promoting survival.

To test whether blockers of electrical activity interfere with the ability of chemical cAMP-elevating agents (*e.g.*, forskolin) to facilitate survival. 10 μM tetrodotoxin (TTX, a voltage-dependent sodium channel blocker) or 5 mM kynurenic acid (KYN, a blocker of ionotropic glutamate receptors) were applied along with forskolin to the cultures, and survival was assessed using the MTT assay at 3 days. Results, shown in Figure 9B, show that neither tetrodotoxin nor kynurenic acid blocked the ability of forskolin to facilitate BDNF and CNTF promoted survival, strongly suggesting that cAMP is the distal effector of electrical activity with respect to its effects on trophic factor-mediated survival.

The protein kinase A inhibitor, Rp-cAMP, was used to determine whether the effects of forskolin were due to the activation (stimulation) of a cAMP-dependent protein kinase in the cells. As shown in Fig. 9A, the enhancement of survival induced either by forskolin or depolarization was completely blocked by 100 μM Rp-cAMP. Note that Rp-cAMP brought the survival level down to the level seen with BDNF alone (the basal survival in the absence of forskolin). Sp-cAMP (100 μM) (an isomer of Rp-cAMP that mimics the effect of forskolin) was tested as a control, and resulted in survival levels similar to those observed with forskolin.

To determine whether glutamate receptor stimulation could also mimic the action of forskolin, the effects of the glutamate receptor agonists n-methyl-d-aspartic acid (NMDA, 10 μM), kainate (100 μM) and quisqualate (100 μM) on the survival of purified P8 RGCs cultured in MBS serum-free medium containing BDNF, CNTF, and insulin were determined. A combination of NMDA and kainate resulted in survival levels almost as high as those facilitated by forskolin. When quisqualate was added along with NMDA and kainate, the survival mimicked the effect of forskolin. Further, the survival effects of the glutamate receptor agonists were also blocked by Rp-cAMP. NMDA, kainate or quisqualate applied individually were less effective. Even at 100 times higher concentrations of NMDA and kainate, no evidence of excitotoxicity (Choi, 1994) was observed after 3 days of culture.

EXAMPLE 7

Morphological and Physiological Properties of Cultured Retinal Ganglion Cells

The morphological and physiological properties of purified retinal ganglion cells in serum-free MBS cultures containing BDNF, CNTF, high insulin, and forskolin were assessed. Figure 10 shown a field of purified postnatal retinal ganglion cells cultured in serum-free MBS medium

containing BDNF, CNTF and IGF-1 for one week. As can be appreciated from the photomicrograph, the cells in such cultures appear healthy, make both dendrites and axons, and show significant dendritic branching.

To assess the expression of retinal ganglion cell markers, 7-day cultures of RGCs were immunostained with an anti-MAP-2 monoclonal antibody (Fig. 11A) or an anti-tau monoclonal antibody (Fig. 11B) as described in Materials and Methods, above. The primary antibody was detected with an FITC-conjugated anti-mouse IgG antibody. The results demonstrate that 7-day cultures of RGCs express the neuronal markers MAP-2 and tau.

The electrical properties of the cultured RGCs were measured as described in Materials and Methods, above, to assess the physiological state of the cells. Purified retinal ganglion cells in the serum-free culture conditions described above fire action potentials in response to depolarizing current injection (Fig. 12A) and exhibit spontaneous excitatory postsynaptic currents (Fig. 12B).

Taken together, the above results indicate that the RGCs maintained in long-term culture under the defined serum-free conditions described herein express the same markers and have the same morphological and physiological properties as freshly-isolated RGCs (Barres, *et al.*, 1988).

EXAMPLE 8

Effects of cAMP Elevation on Survival of Purified Cortical Neurons in Culture

Thy-1 positive cerebral cortical neurons were purified by immunopanning from postnatal day 8 rat cerebral cortices as described above. The cells were plated in MBS medium containing BDNF, CNTF, insulin and forskolin, individually or in combination as indicated, and their survival was assessed using the MTT assay three days after plating.

Exemplary results are shown in Figure 13. The growth factors added (concentrations as above) are indicated below each data set. The presence and absence of forskolin is indicated by (+) and (-), respectively. In the absence of forskolin, the number of surviving cells was very low. Single factors applied individually (BDNF, CNTF) resulted in less than 5% survival. Even when applied together, and in the presence of insulin, the survival was less than about 15%. In contrast, when forskolin was present in the culture medium, the number of cells surviving under each set of conditions was increased by about 6 to 10 -fold.

Further, the morphological characteristics of the cells in each of the samples roughly paralleled the number of cells surviving. That is, the higher the rate of survival, the more differentiated the cells appeared under the microscope. In particular, cells in the dish containing BDNF, CNTF, insulin and forskolin were very well differentiated, typically having large somas and displaying several dendrites and one axon per cell.

EXAMPLE 9

Characterization of Oligodendrocyte-Derived RGC Survival Factor

Serum-free MBS medium containing CNTF (50 ng/ml) and high insulin (5 μg/ml) was conditioned by a dense culture of mature oligodendrocytes for at least three days. To determine whether the non-CNTF, non-insulin survival activity was a protein, a sample of the conditioned medium was heat inactivated by boiling for 5 minutes. Another sample of the medium (1 ml) was incubated for 16 hours at 37°C with beads spun down from 0.5 ml of a solution of trypsin-coupled beads (Pierce Chem. Co., Rockford, IL). As a control, medium lacking beads was incubated in parallel. The trypsin digestion was terminated by centrifugation at 1000 *g* for 10 minutes to separate the beads from the supernatant.

To assess whether the size of the unknown factor was greater than 10 kD, the oligodendrocyte conditioned medium was centrifuged through a "CENTRIPREP-10" concentrator membrane (Amicon, Beverly, MA), which excludes molecules greater than 10 kD, at 2500 *g* for 50 min. This manipulation resulted in a 10-fold concentration of the medium components > 10 kD. The ability of a 20-fold dilution of the concentrated medium (containing activities greater than 10 kD) and a 1:1 dilution of the filtrate (containing activities less than 10 kD) to promote survival was assessed as follows.

Approximately 5,000 purified P8 retinal ganglion cells were plated in triplicate onto merosin-coated wells in a 96-well plate (Falcon) in 100 μl of MBS medium containing high insulin (5 μg/ml), forskolin (5 μM), BDNF (50 ng/ml), and CNTF (50 ng/ml). The medium was then diluted 1:1 with the indicated oligodendrocyte conditioned medium that had been treated as indicated. The cells were incubated in this medium for 5 days.

The results are shown in Table 5, below.

Table 5

EFFECTS OF GLIAL CELL CONDITIONED MEDIUM ON THE SURVIVAL OF PURIFIED RETINAL GANGLION CELLS IN CULTURE

Conditioning Medium (CM) Treatment	% Surviving Cells Forskolin+BDNF+CNTF +INS
No Conditioned Medium	34.4 ± 0.5
Oligo CM	48.8 ± 5.2
Heat Inactivated Oligo CM	33.4 ± 1.2
Trypsin Digested Oligo CM	26.2 ± 1.0
Oligo CM, Fraction > 10kD	50.2 ± 2.7
Oligo CM, Fraction < 10kD	28.5 ± 0.5

The above results suggest that oligodendrocyte-derived neurotrophic factor (ODNF) is a trypsin-sensitive heat-sensitive > 10 kD proteinaceous factor secreted by oligodendrocytes into the bathing medium.

EXAMPLE 10

Cloning of Oligodendrocyte-Derived RGC Survival Factor

A cDNA library was prepared from highly purified (> 99.95% pure) mature oligodendrocytes in the expression vector pMET7 (Takabe, 1988), which contains the SR alpha promoter, the T7 polymerase binding site 5' to a polylinker modified for *NotI*/*SalI* directional insertion of cDNA, and the SV40 late-gene splice junction. The SR alpha promoter contained in the vector is up to 100 times more efficient in driving the expression of exogenously introduced genes compared to unmodified SV40 early promoter constructs (Kriegler, 1990).

Total RNA was prepared from cultures of ~100 million purified mature oligodendrocytes by the single step guanidinium isothiocyanate method of Chomczynski and Sacchi (1987). Poly-A^+ mRNA was purified from the total RNA using an oligo-dT matrix ("OLIGOTEX", Qiagen Inc., Chatsworth, CA) according to the manufacturer's instructions. First and second strand cDNA were synthesized using the "SUPERSCRIPT" Choice cDNA synthesis kit (Gibco BRL), which utilizes a *NotI* oligo-dT primer for directional cloning and "SUPERSCRIPT" II RNAse H- reverse transcriptase, following manufacturer's instructions.

The cDNA fragments were ligated to *SalI* linkers, digested with *NotI* and size selected over a "SEPHACRYL" S-500 column (Promega Corp., Madison, WI; "SEPHACRYL" is a suspension of particles of crosslinked co-polymer of allyl dextran and N,N'-methylenebisacrylamide in ~20% ethanol). Fragments greater than 1 kb were directionally ligated into *NotI* and *SalI* sites in the pMET7 vector, and the library was electroporated into electrocompetent DH12S cells (Gibco/BRL). A characterization of the library revealed over one million independent recombinants with an average insert size 1.6 kB. Two of 8 clones analyzed had insert sizes of over 3.0 kB.

The library was amplified and divided into pools, from which groups of plasmids were isolated for transfection using lipofectamine (or DEAE Dextran) into COS7 cells.

Two days after transfection, conditioned medium from the transfected cells is collected and assayed for survival-promoting activity (as described above) on purified postnatal RGCs. Conditioned medium from untransfected COS7 cells is tested to verify that the COS7 cells do not produce any factors that enhance survival of purified RGCs. Pools of cells that enhance survival are then subcloned and rescreened until individual clones having survival activity are identified.

EXAMPLE 11

Characterization of Clones Encoding Oligodendrocyte-Derived Neurotrophic Factor

cDNA clones encoding the RGC survival activity (ODNF) are isolated, sequenced, and the sequences compared for homologies to known sequences. Northern blot and *in situ* hybridization analyses are performed to verify that the mRNAs are expressed specifically by oligodendrocytes but not astrocytes.

A clone encoding the full-length ODNF is used to prepare a fusion protein for the production of recombinant survival factor polypeptide. The coding sequences are cloned into an expression plasmid, such as pGEX, to produce corresponding polypeptides. The recombinant pGEX plasmid is transformed into an appropriate strains of *E. coli* and fusion protein production is induced by the addition of IPTG (isopropyl-thio galactopyranoside). Solubilized recombinant fusion protein is then purified from cell lysates of the induced cultures using glutathione agarose affinity chromatography according to standard methods (Ausubel, *et al.*).

EXAMPLE 12

Regeneration of Optic Nerve Following Treatment with Oligodendrocyte-Derived Neurotrophic Factor

The rat optic nerve is surgically cut or crushed retroorbitally while the rat is under inhalation anesthesia as previously described (Berkelaar, *et al.*, 1994). This procedure avoids injury to major retinal blood vessels and enables assessment of the amount of regrowth of neurofilament-positive axons into the optic nerve. The amount of regeneration is measured by removing the optic nerves after a regeneration period of two weeks following P8 optic nerve transection.

P8 animals are used in the initial studies because previous experiments have established that it is possible to successfully deliver high levels of trophic factors into the optic nerves and retina at this age (see below). The experiments are then extended to include older rats. One group of animals is treated with intraocular injections of ODNF, anther group with intracerebral injections, and control groups of animals receive sham injections.

The amount of regeneration is assessed by sectioning and labeling with neurofilament antibodies. A quantitative assessment of the amount of regeneration (how many axons and how far they regenerated) is obtained by cutting the optic nerves into three pieces (retinal, middle, and chiasmal), using an immunoenzyme assay to measure the total content of neurofilament protein, and comparing this to control untransected nerves.

EXAMPLE 13

Delivery of Growth Factors to the CNS

A. Direct Injection

Growth factors, forskolin and IBMX were injected into rat eyes *in vivo* by intraocular injection, according to methods established in the art. Target concentrations of about 50 ng/ml growth factor(s), 0.1 mM IBMX and 5 μM forskolin were maintained by initial injections of 100x these concentrations, in order to compensate for leakage of the compounds out of the retinas over the three day exposures.

Similar methods are used to inject growth factors, activators of adenylate cyclase and/or protein kinase and phosphodiesterase inhibitors into other regions of the central nervous system. For example, injections to the spinal cord following lesioning can be carried out as described by Schnell, *et al.* (Nature 367: 170-173, 1994; incorporated herein by reference. For example, using a rat model, NT-3 (300-500 μg in 0.3-0.5 μl) is co-injected with forskolin and possibly IBMX immediately rostral to a lesion in the spinal cord of young rats (4-7 week old). Regeneration is assessed by immunostaining, according to methods known in the art.

B. Transplantation of Growth-factor producing Cells

Stably-transfected cell lines secreting RGC survival factors are transplanted into the subarachnoid space of P8 rats as previously described (Barres, *et al.*, 1992, 1993, 1993a, 1994; Barres and Raff, 1993). After 2 days, the optic nerves of 4 "test" groups of animals are transected or crushed, while those of 4 "control" groups are left intact. The animals are maintained for 10 days following nerve transection and are then sacrificed using halothane anesthesia.

The optic nerves from each of the 4 control and test groups are fixed in 4% paraformaldehyde, cryosectioned and stained with neurofilament antibodies to assess whether regeneration has occurred. The factor-secreting cell lines that are used include 293 cells that secrete human BDNF (homologous to rat BDNF) and 293 cells that release a secretable form of rat CNTF, that has been engineered to contain the secretory signal sequence of human growth factor.

Stably transfected 293 cells containing recombinant ODNF are also prepared and used to assess the effects of this factor on RGC survival. Combinations of recombinant factors are also evaluated as describe above. The levels of trophic factor delivered into the optic nerve are assessed by enzyme-linked immunesorbent assay (ELISA). The optic nerve is removed from the meningeal sleeve for this analysis.

The experiment described above are conducted both in the presence and absence of intraocular injection of forskolin, to evaluate the *in vivo* effectiveness of the drug. The injections are repeated every 4 days throughout the postoperative regeneration period.

EXAMPLE 14

Stimulation of cAMP production in Neuronal Cells

A. Activation of Adenylate Cyclase

Cultures of purified retinal ganglion cells were exposed to 0, 1 and 10 μM forskolin, an activator of adenylate cyclase, for 15 minutes prior to immunostaining with an anti-cAMP antibody according to established procedures (see Materials and Methods, above). Results are shown in Figures 14A-14C, where it is apparent that forskolin treatment results in concentration-dependent increases in intracellular cAMP. Parallel results were observed using a cAMP radioimmunoassay on the cells.

B. Depolarization

Purified retinal ganglion cells in culture were exposed for 15 minutes to no treatment, 0.1 mM IBMX alone, or depolarizing conditions: glutamate + IBMX or 10 mM KCl+IBMX. Results are shown in Figure 15, where both glutamate and high KCl treatments resulted in increased intracellular concentrations of cAMP, as assessed by immunofluorescence (anti-cAMP antibody).

EXAMPLE 15

Nuclear Translocation of MAP Kinase (erk1)

Retinal ganglion cells in separate culture dishes were exposed to growth factors in the absence or presence of forskolin. Nuclear translocation of MAP kinase was assessed by immunofluorescence staining of cells using as primary antibody anti-MAP kinase (see Materials and Methods, above), and quantitating fluorescence present in a nuclear fraction. Results are shown in Figure 16, where the number of cells with translocated MAP kinase is indicated on the abscissa as a function of time. Lines labeled "cAMP" are plots obtained from cells exposed to 5 μM forskolin. As shown, the combination of BDNF and forskolin resulted in a marked enhancement of MAP kinase translocation.

EXAMPLE 16

In vivo Activation of Adenylate Cyclase

Retinal experiments were carried out by injecting substances directly into the vitreous fluid of the rat eye, or by excising whole rat retina by dissecting the retinas into a dish with basal medium. Figure 17 shows the results of experiments in which whole retinas were exposed to 0.1 mM IBMX alone or IBMX with forskolin (5 μM), then tested for cAMP levels using anti-cAMP antibodies, as described above. In prior experiments (not shown) lower levels of activation were observed when forskolin was used alone.

EXAMPLE 17

In vivo Nuclear Translocation of MAP kinase

P8 retinas were incubated for three hours in serum-free medium in the absence or presence of a cocktail of growth factors BDNF and CTNF (50 ng/ml each), forskolin (5μM) and IBMX (0.1 mM) were sectioned and stained with anti-MAP kinase antibody. Results are shown in Figure 18, where panels A and B show control retinas with few immunoreactive nuclei, while panels C and D show cocktail treated retinas where most nuclei are immunoreactive. Further quantitation of this phenomenon is shown in Figure 19, where "Factors" indicates retinas treated with BDNF and CTNF (50 μg/ml each), and "cAMP" indicates retinas treated with 5 μM forskolin in the presence of IBMX (0.1 mM). In this experiment, blockers of depolarization (tetrodotoxin (TTX), curare, and kynurenic acid (Kyn) were able to reverse the translocation stimulatory effects of the growth factor/forskolin cocktail.

EXAMPLE 18

In vivo Survival of CNS Cells under Degenerating Conditions

In vivo retinal studies were carried out on anesthetized rats. P8 retinal ganglion cells were retrogradely labelled using fluorogold *in vivo* in rat retinas. The retinal ganglia were then transected retroorbitally taking care to preserve retinal circulation followed by intraocular injection of cocktails containing vehicle, BDNF, a cocktail of BDNF, CNTF and IGF-1 ("factors" in Figure 20), a cocktail of the same factors + forskolin, or a cocktail of factors, forskolin + IBMX. Initial intraorbital concentration targets were approximately 100x the concentrations usually used in *in vitro* experiments, to compensate for leakage over several days: *e.g.*, 5μg/ml growth factors, 500 μM forskolin, 10 mM IBMX. Retinal ganglion cell survival was measured after 3 days by determining cell density in the central retina. As shown in Figure 20, few cells survived when the vitreous was injected with a single trophic factor alone (BDNF), whereas retinal ganglion cell survival was significantly enhanced by treatment with BDNF, CNTF, and

IGF-1 together and was further enhanced when cAMP was also elevated by forskolin and IBMX. Figure 21 shows computer-generated images of fluorogold-labeled retinal ganglion cells in the absence (panel A) or presence (panels B and C) of degenerating conditions (axotomy ("cut"), as described above).

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While the invention has been described with reference to specific methods and embodiments, it is appreciated that various modifications and changes may be made without departing from the invention. All references cited are hereby incorporated by reference herein in their entireties.

IT IS CLAIMED:

1. A method of promoting survival of neuronal cells in the central nervous system in a subject, comprising

exposing said cells to (i) a growth factor selected from the group consisting of insulin-like growth factors, neurotrophins, and CNTF-like cytokines, and (ii) conditions effective to produce elevated levels of cyclic adenosine monophosphate (cAMP) in said cells, as evidenced by ability to stimulate a cAMP-dependent protein kinase in said cells.

2. The method of claim 1, wherein said growth factor comprises a plurality of growth factors.

3. The method of claim 1, wherein said growth factor is selected from the group consisting of insulin, brain-derived neurotrophic factor (BDNF), insulin-like growth factor 1 (IGF-1), insulin-like growth factor 2 (IGF-2), neurotrophin-4/5 (NT-4/5), ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), interleukin-6 (IL-6), basic fibroblast growth factor (bFGF), and transforming growth factor-alpha (TGF-α).

4. The method of claim 1, wherein said exposing is accomplished by contacting said cells with a growth factor and an activator of a cAMP dependent protein kinase.

5. The method of claim 4, wherein said protein kinase activator is selected from the group consisting of forskolin, chlorophenylthio-cAMP (CPTcAMP), Sp-cAMP and 8-bromo cAMP.

6. The method of claim 5, wherein said activator is forskolin.

7. The method of claim 4, wherein said contacting further includes contacting said cells with an inhibitor of phosphodiesterase (PDE).

8. The method of claim 7, wherein said PDE inhibitor is isobutylmethylxanthine (IBMX).

9. The method of claim 4, wherein said contacting is by injection into an extracellular region surrounding said cells.

10. The method of claim 9, wherein said cells are retinal ganglion cells, and said injection of growth factor and adenylate cyclase activator is accomplished by intraocular injection.

11. The method of claim 10, wherein said growth factor includes one or more of the group consisting of brain-derived neurotrophic factor (BDNF), insulin-like growth factor 1 (IGF-1) and ciliary neurotrophic factor (CNTF), and wherein said conditions effective to produce elevated levels of cAMP include exposure of said cells to a phosphodiesterase inhibitor.

12. The method of claim 1, wherein said conditions effective to activate adenylate cyclase include depolarizing said cells by electrical stimulation.

13. The method of claim 1, wherein said activation of adenylate cyclase is evidenced by activation of MAP-kinase.

14. The method of claim 13, wherein said activation of MAP kinase is evidenced by translocation to the nucleus of said kinase.

15. An *in vitro* culture of central nervous system (CNS) neuronal cells, said cells characterized by extended viability time in culture as defined by maintenance of neuronal cell characteristics and at least 30% cell survival after 3 days of culture,
comprising

isolated central nervous neuronal cells, said cells cultured

(i) in substantially serum-free medium;

(ii) in the presence of a growth factor selected from the group consisting of insulin-like growth factors, neurotrophins, and CNTF-like cytokines;
and

(iii) under conditions effective to produce elevated levels of cyclic adenosine monophosphate (cAMP) in said cells, as evidenced by ability to stimulate a cAMP-dependent protein kinase in said cells.

16. The cell culture of claim 15, wherein said culture conditions are further characterized by an absence of feeder cells or medium conditioned by feeder cells.

17. The culture of claim 15, wherein said CNS neuronal cells are prenatal neuronal cells.

18. The culture of claim 15, where said CNS neuronal cells are postnatal neuronal cells.

19. The culture of claim 18, wherein said postnatal CNS neuronal cells are further characterized by long-term viability, as defined by:

(i) at least 30% cell survival after 7 days of culture, and

(ii) maintenance of cell characteristics, as evidenced by the presence of a measurable cell-specific marker in the surviving cells after 7 days.

20. The culture of claim 19, further characterized by very long-term viability, as defined by at least 30% cell survival after 28 days of culture.

21. The culture of claim 15, wherein said maintenance of neuronal cell characteristics is evidenced by the presence of a measurable cell-specific marker in the surviving cells.

22. The culture of claim 21, wherein said cell-specific marker is selected from the group consisting of MAP-2 and tau.

23. The culture of claim 15, wherein said CNS neuronal cells are selected from the group consisting of glutamatergic projection neurons, cortical glutamatergic projection neurons and retinal ganglion cells.

24. A method of growing an *in vitro* culture of central nervous system (CNS) neuronal cells characterized by extended survival time in culture, comprising

incubating said cells in a medium which

(i) is substantially free of feeder cells or medium conditioned by feeder cells,

(ii) is substantially serum-free, and

(iii) contains a growth factor effective to increase survival time of said cells, and

growing said cells under conditions effective to stimulate cAMP-dependent protein kinase in said cells.

25. The method of claim 24, wherein said growth factor is selected from the group consisting of

insulin-like growth factors, neurotrophins and CNTF-like cytokines.

26. The method of claim 25, wherein said growth factor is selected from the group consisting of insulin, brain-derived neurotrophic factor (BDNF), insulin-like growth factor 1 (IGF-1), insulin-like growth factor 2 (IGF-2), neurotrophin-4/5 (NT-4/5), ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), interleukin-6 (IL-6), basic fibroblast growth factor (bFGF), and transforming growth factor-alpha (TGF-α).

27. The method of claim 24, wherein said conditions effective to stimulate a cAMP-dependent protein kinase include exposing said cells to an activator of adenylate cyclase selected from the group consisting of forskolin, chlorophenylthio-cAMP (CPTcAMP), Sp-cAMP and 8-bromo cAMP.

28. The method of claim 24, wherein the conditions effective to stimulate a cAMP-dependent protein kinase include depolarizing said cells in the presence of about 200 μM to 10 mM extracellular calcium.

29. The method of claim 28, wherein said depolarizing is accomplished by exposing said cells to a glutamate receptor agonist.

30. The method of claim 28, wherein said depolarizing is accomplished by electrically stimulating said cells.

31. The method of claim 24, wherein said medium includes a plurality of growth factors selected from the group consisting of insulin-like growth factors, neurotrophins, and CNTF-like cytokines.

32. A pharmaceutical composition for use in promoting survival of central nervous system neurons in a subject, comprising

a growth factor selected from the group consisting of insulin-like growth factors, neurotrophins, and CNTF-like cytokines,

means for elevating intracelluar levels of cAMP, and

a suitable pharmaceutical excipient.

33. The pharmaceutical composition of claim 32, wherein said means for elevating cAMP levels is selected from the group consisting of forskolin and membrane permeable cAMP analogs.

34. The pharmaceutical composition of claim 32, wherein said growth factors comprises a plurality of growth factors selected from the group consisting of insulin, brain-derived neurotrophic factor (BDNF), insulin-like growth factor 1 (IGF-1), insulin-like growth factor 2 (IGF-2), neurotrophin-4/5 (NT-4/5), ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), interleukin-6 (IL-6), basic fibroblast growth factor (bFGF), and transforming growth factor-alpha (TGF-α).

35. The pharmaceutical composition of claim 32, which further comprises an inhibitor of phosphodiesterase.

36. The pharmaceutical composition of claim 35, wherein said phosphodiesterase inhibitor is isobutylmethylxanthine (IBMX).

37. The pharmaceutical composition of claim 32, wherein said means to elevate cAMP levels is provided by depolarizing means.

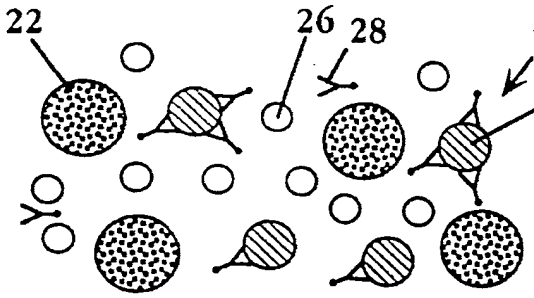
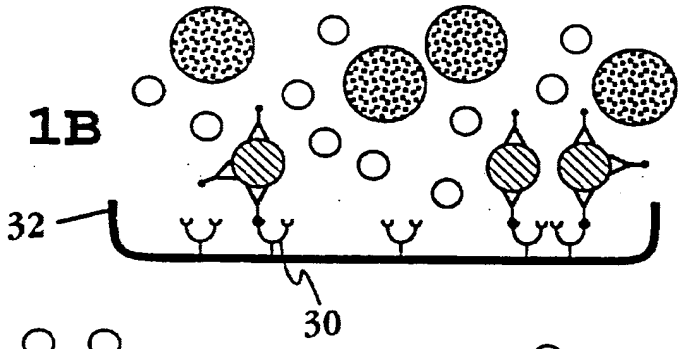


Fig. 1A

Fig. 1B



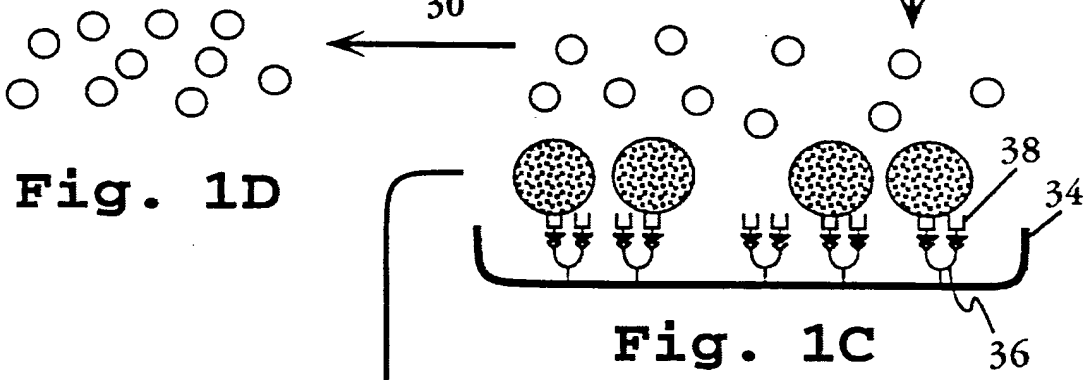


Fig. 1D

Fig. 1C

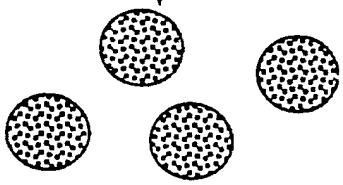


Fig. 1E

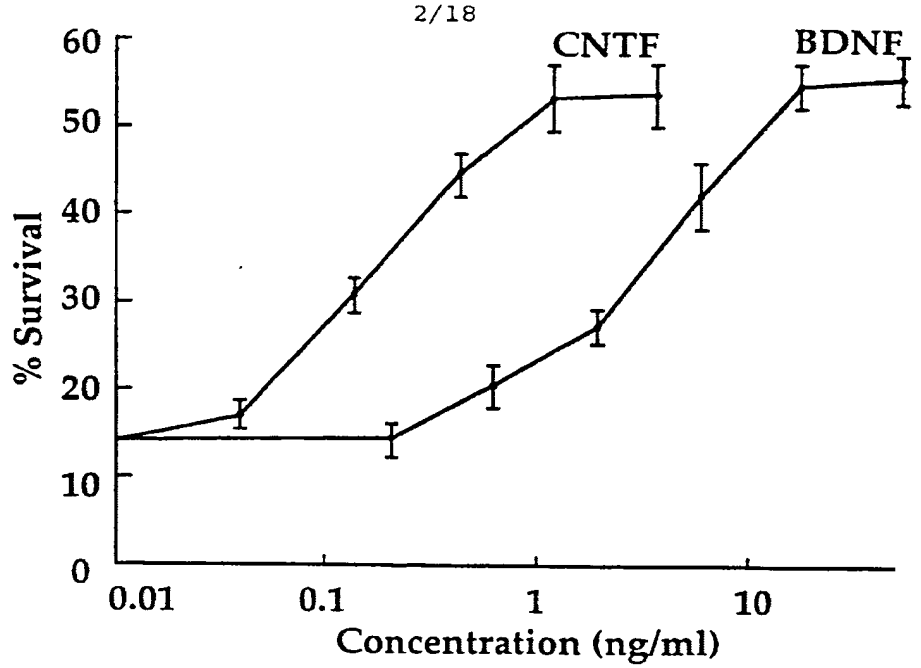

CNTF
BDNF
% Survival
60
50
40
30
20
10
0
0.01
0.1
1
10
Concentration (ng/ml)

Fig. 2A

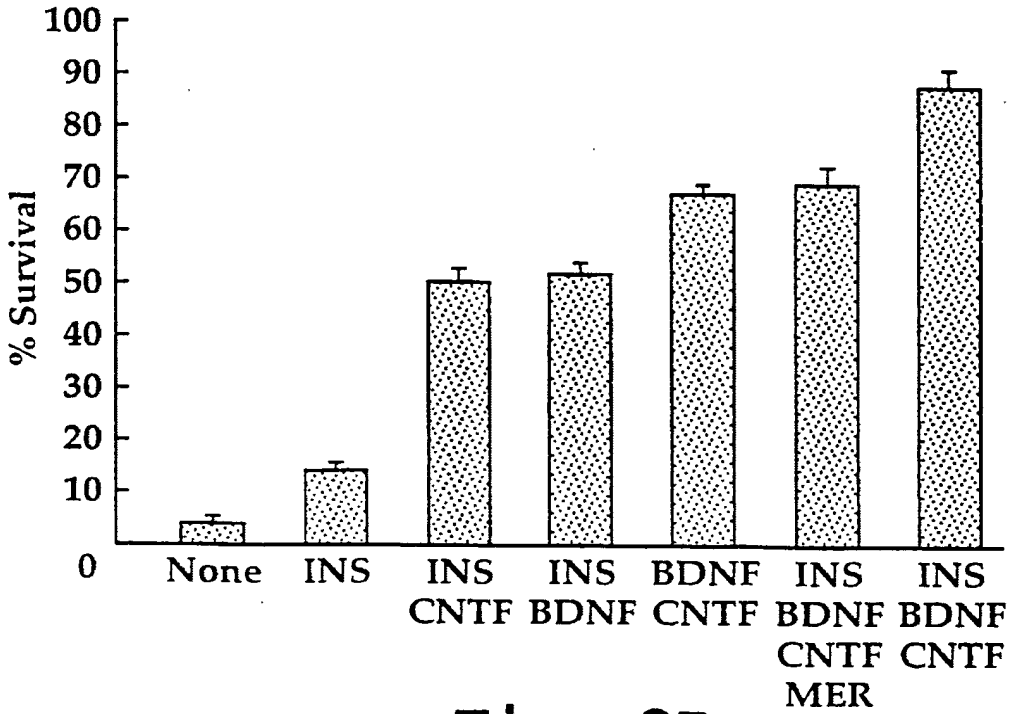

% Survival
100
90
80
70
60
50
40
30
20
10
0
None
INS
INS CNTF
INS BDNF
BDNF CNTF
INS BDNF CNTF MER
INS BDNF CNTF

Fig. 2B

Fig. 3

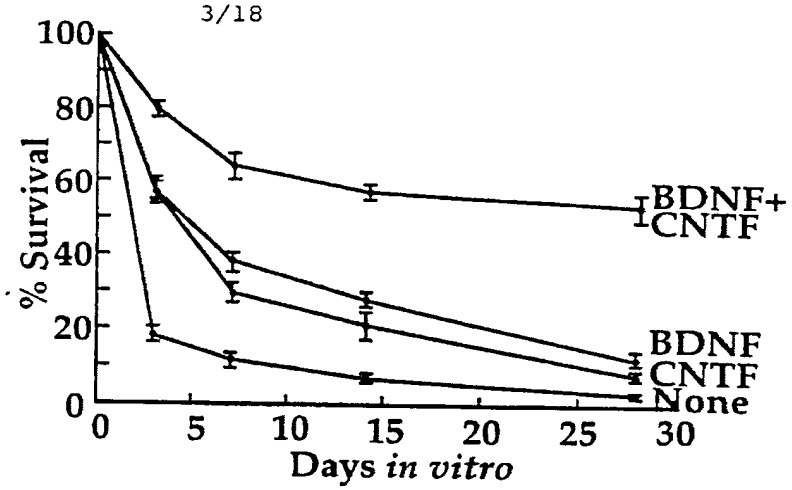


Fig. 4A

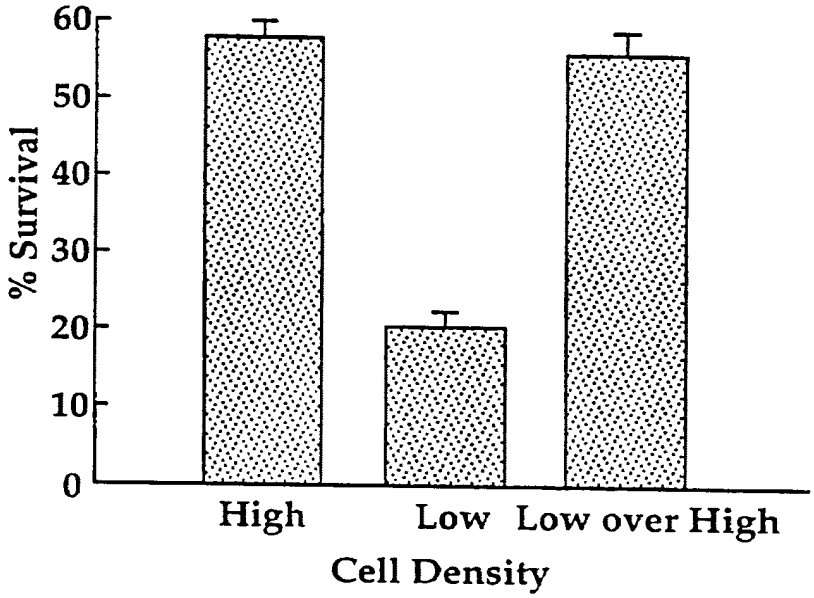


Fig. 4B

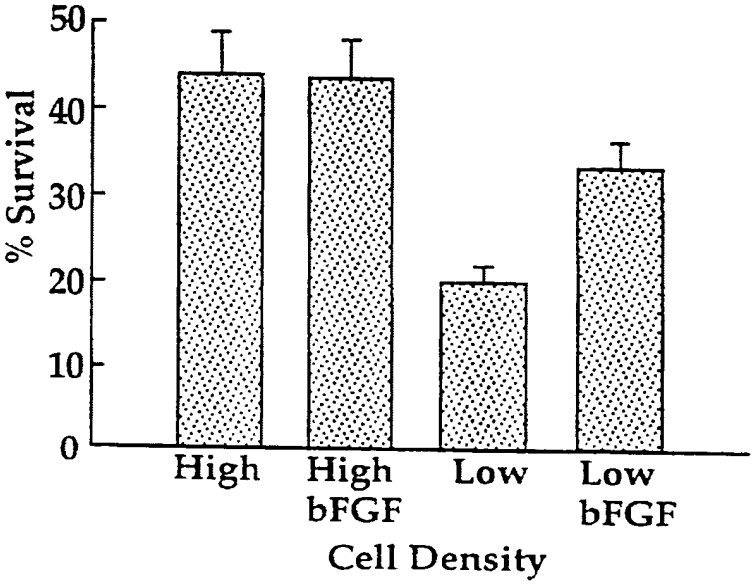


Fig. 5A

Fig. 5B

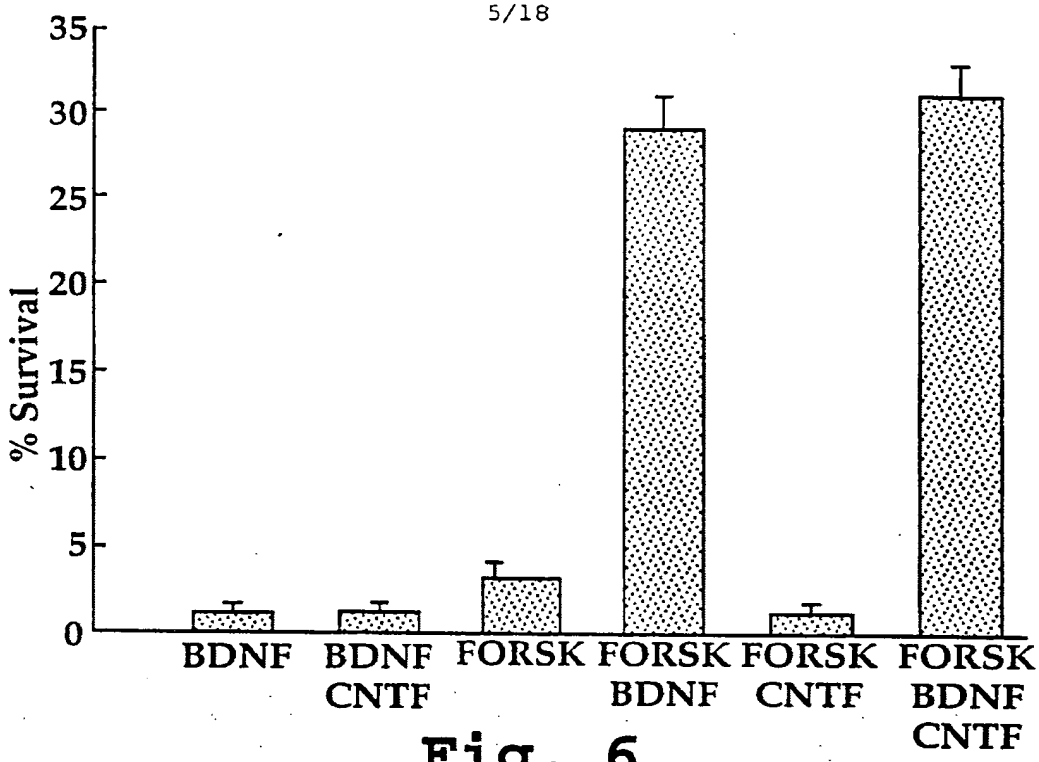

35
30
25
20
15
10
5
0
% Survival
BDNF
BDNF CNTF
FORSK
FORSK BDNF
FORSK CNTF
FORSK BDNF CNTF

Fig. 6

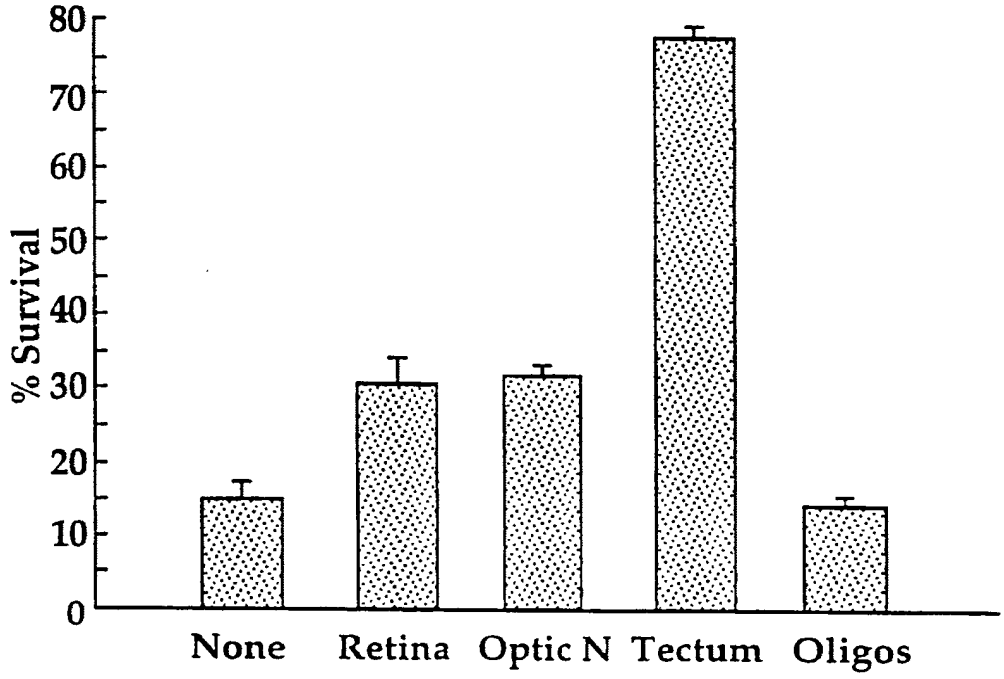

80
70
60
50
40
30
20
10
0
% Survival
None
Retina
Optic N
Tectum
Oligos

Fig. 7

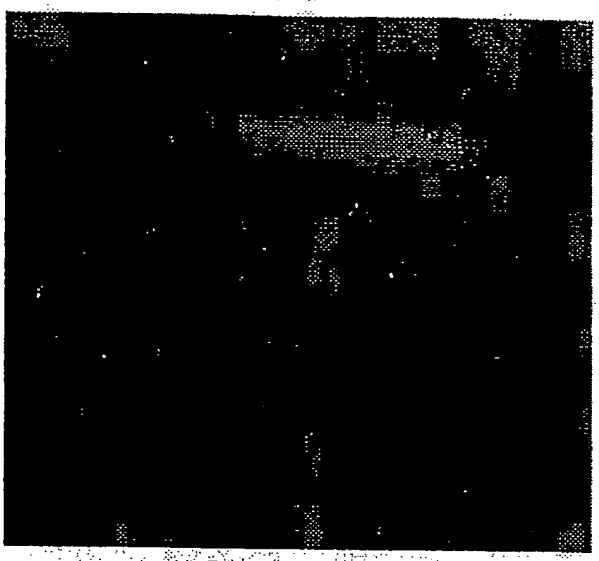


Fig. 8A

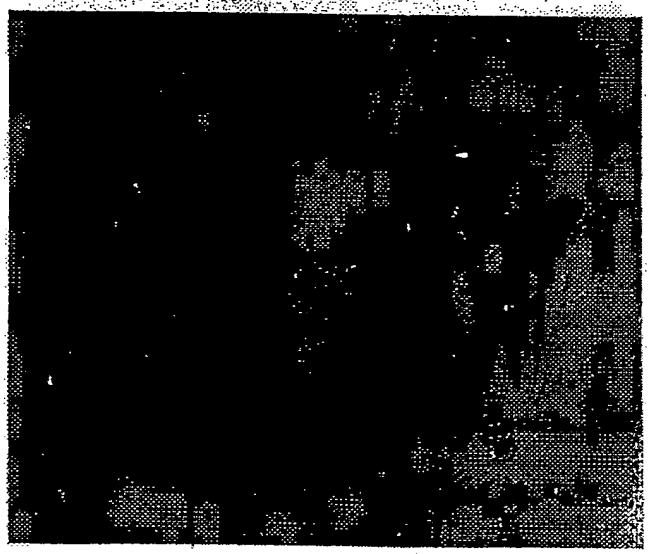


Fig. 8B

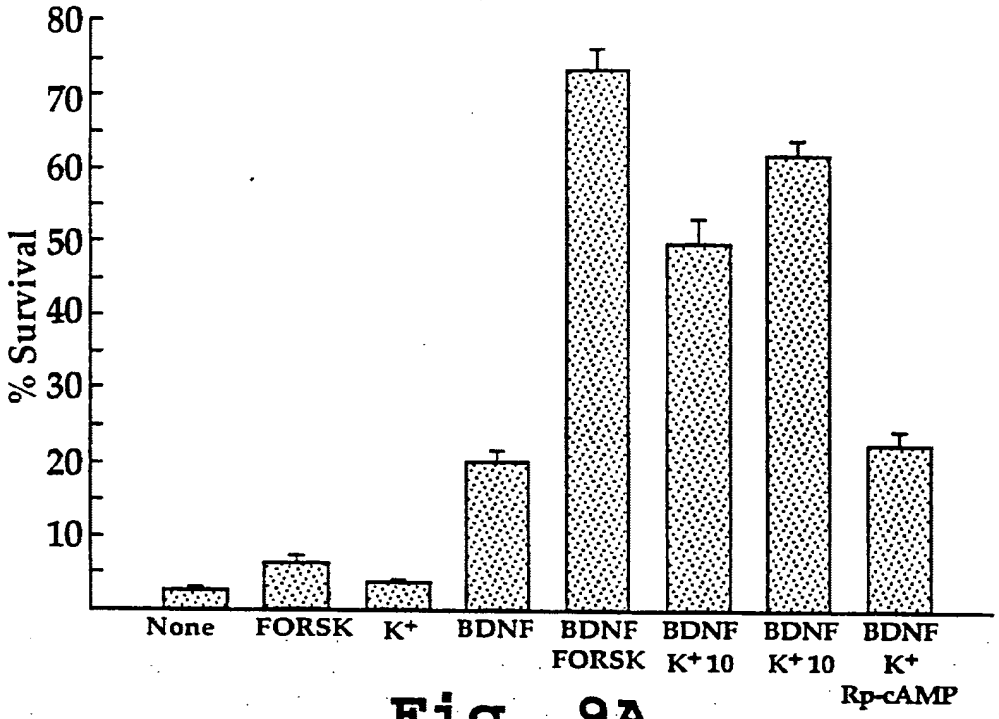

% Survival
80
70
60
50
40
30
20
10
None
FORSK
K+
BDNF
BDNF FORSK
BDNF K+10
BDNF K+10
BDNF K+ Rp-cAMP

Fig. 9A

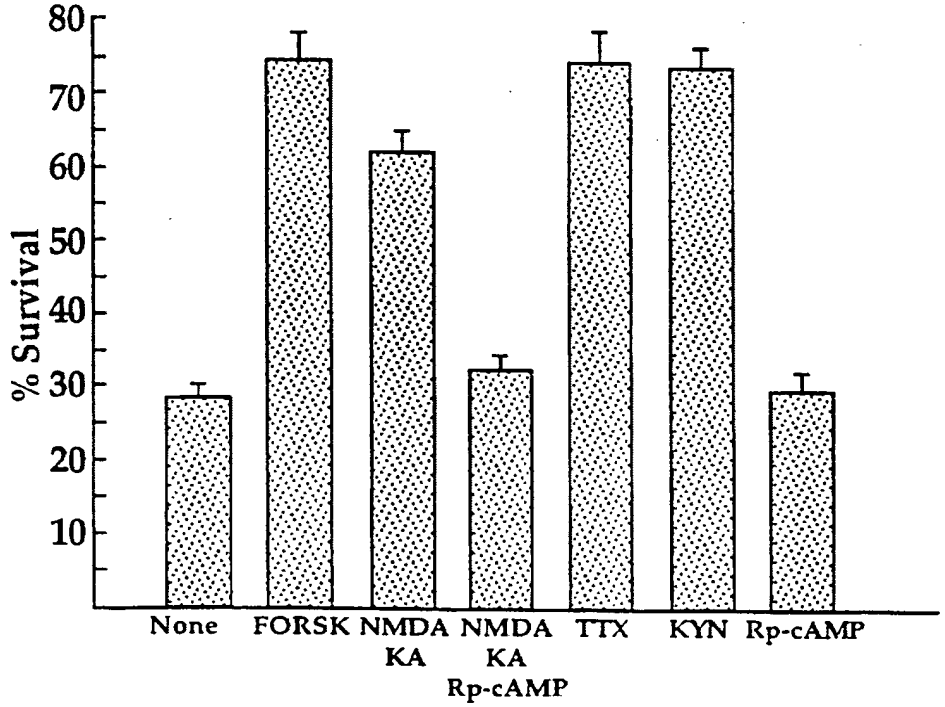

% Survival
80
70
60
50
40
30
20
10
None
FORSK
NMDA KA
NMDA KA Rp-cAMP
TTX
KYN
Rp-cAMP

Fig. 9B

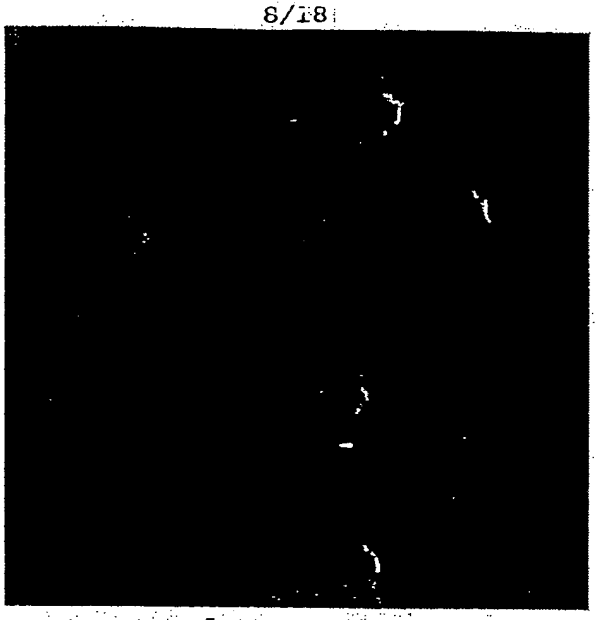


Fig. 10

Fig. 11A

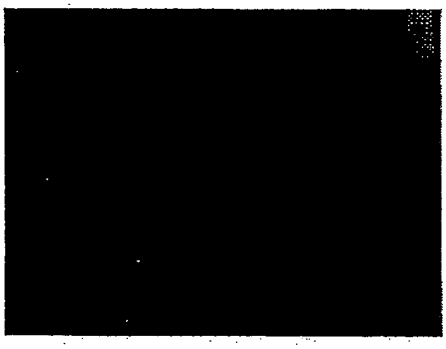


Fig. 11B

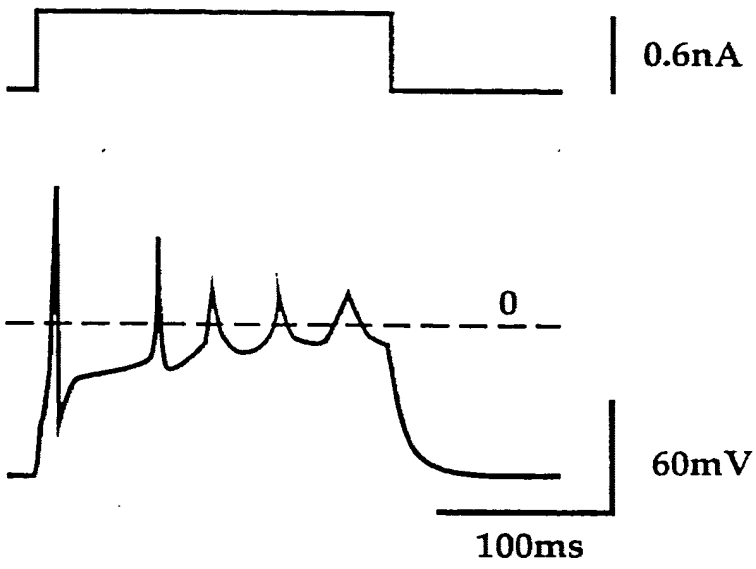


Fig. 12A

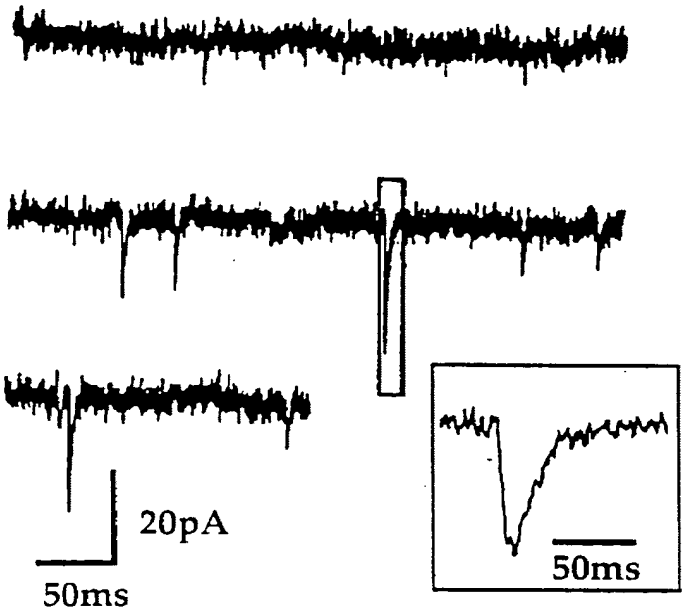


Fig. 12B

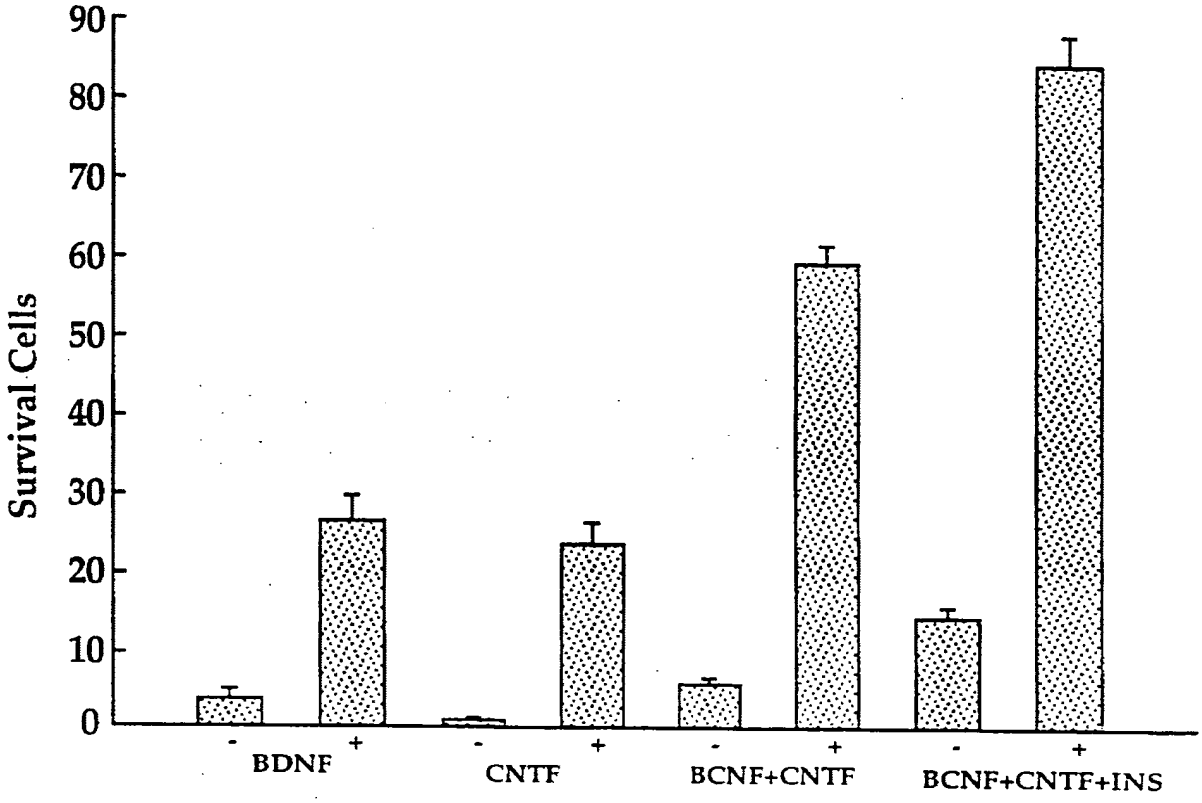
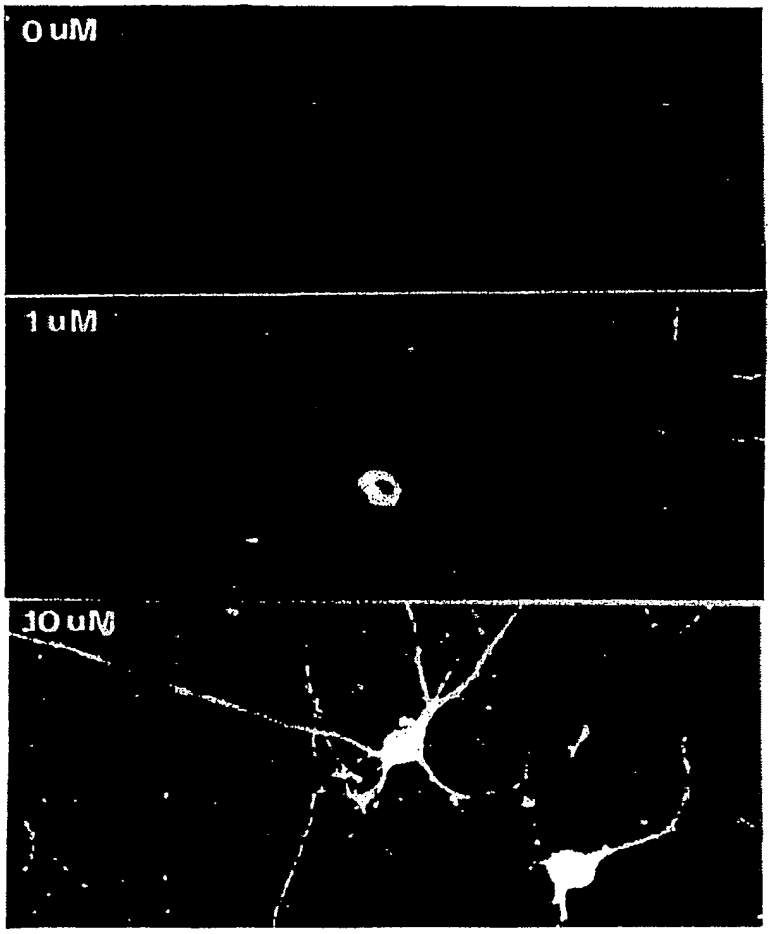

Survival Cells
90
80
70
60
50
40
30
20
10
0
-
+
BDNF
CNTF
BCNF+CNTF
BCNF+CNTF+INS

Fig. 13

Fig. 14A

Fig. 14B

Fig. 14C



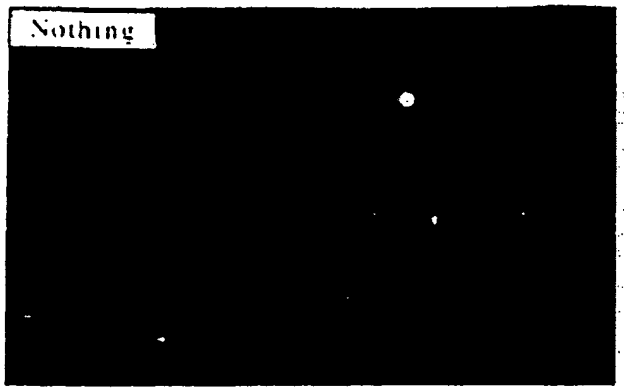


Fig. 15A

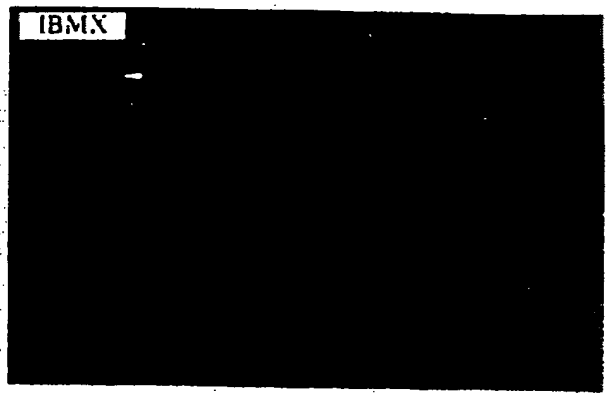


Fig. 15B

Fig. 15C

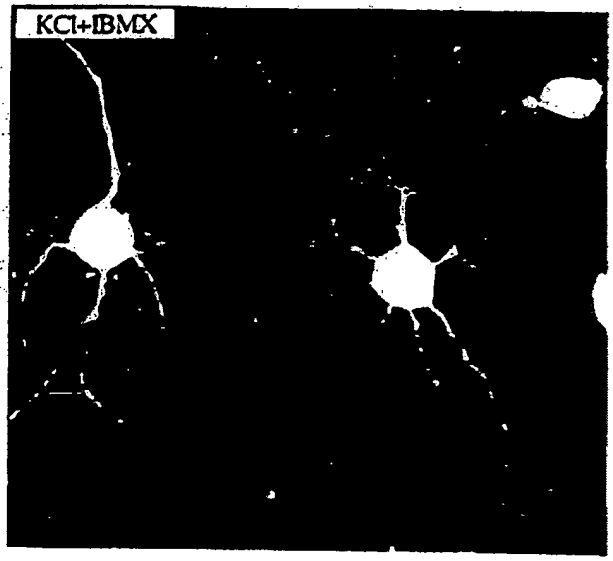


Fig. 15D

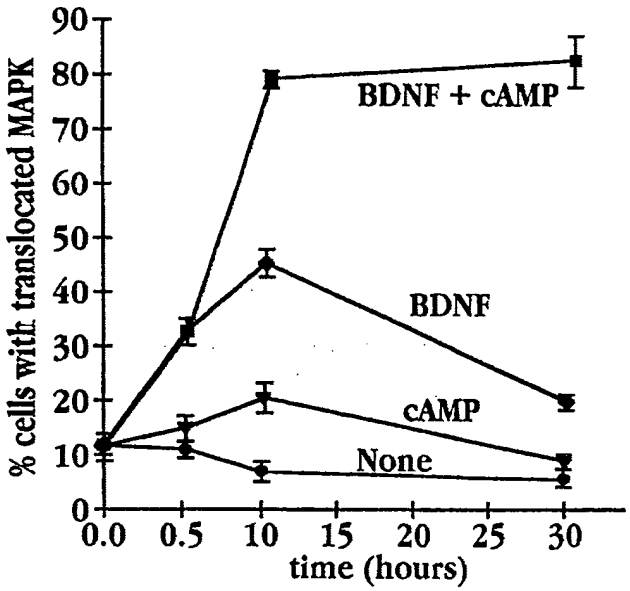

90
80
70
60
50
40
30
20
10
0
% cells with translocated MAPK
BDNF + cAMP
BDNF
cAMP
None
0.0
0.5
10
15
20
25
30
time (hours)

Fig. 16

Fig. 17A

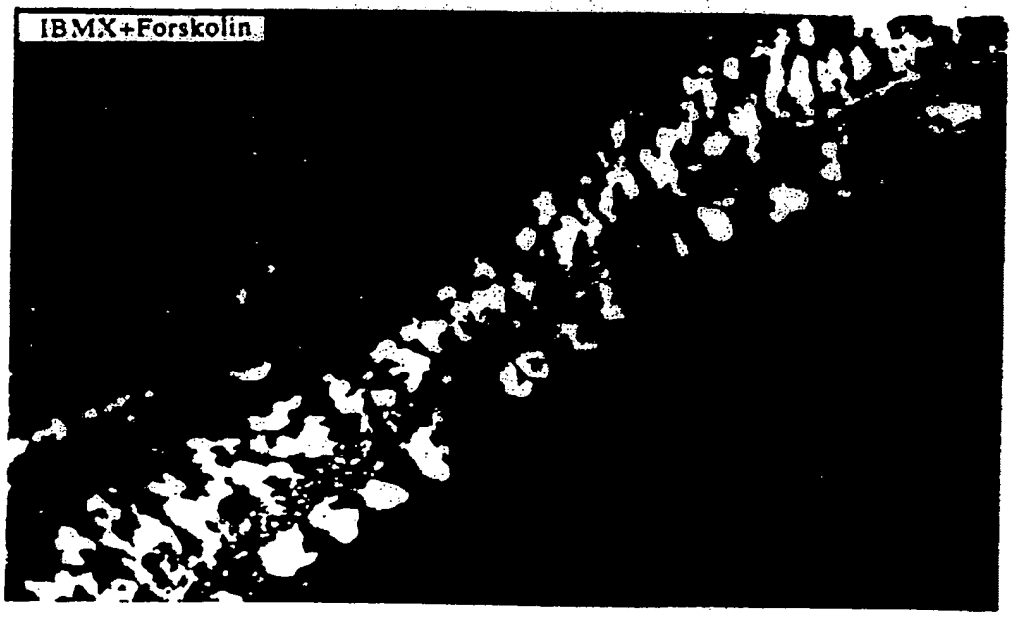


Fig. 17B

Fig. 18A

Fig. 18B

Fig. 18C

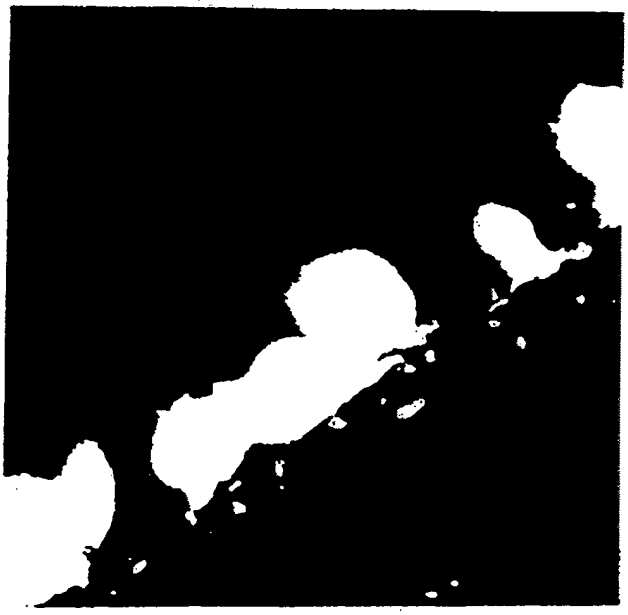


Fig. 18D

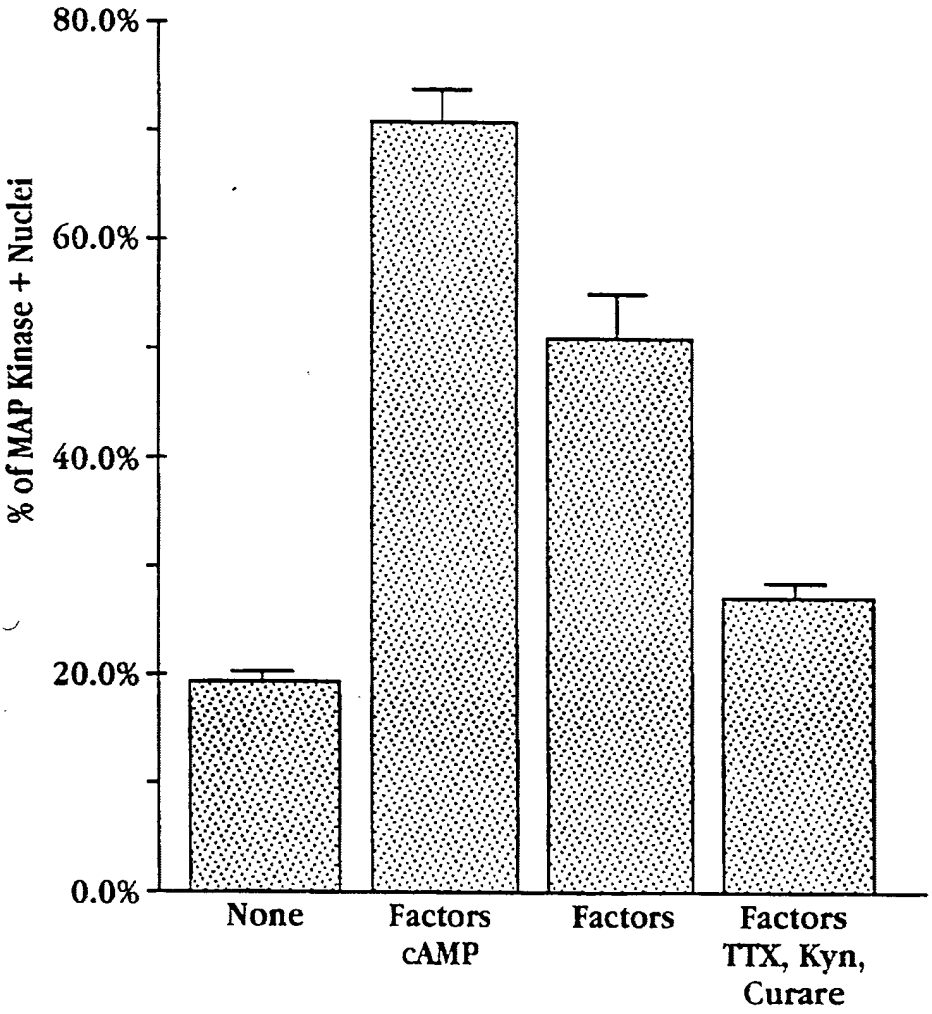

80.0%
60.0%
40.0%
20.0%
0.0%
% of MAP Kinase + Nuclei
None
Factors
cAMP
Factors
Factors
TTX, Kyn,
Curare

Fig. 19

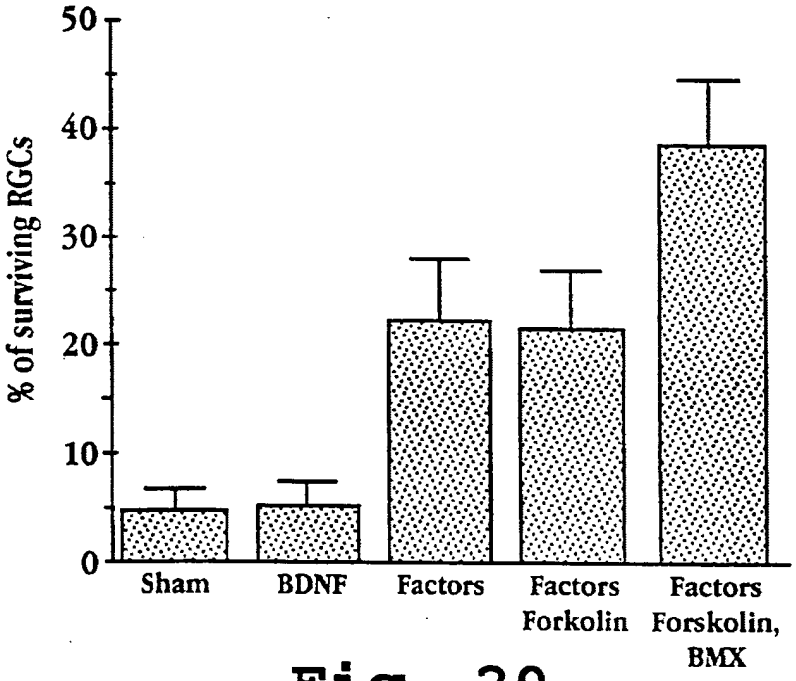

50
40
30
20
10
0
% of surviving RGCs
Sham
BDNF
Factors
Factors Forkolin
Factors Forskolin, BMX

Fig. 20

Fig. 21A

Fig. 21B

Fig. 21C